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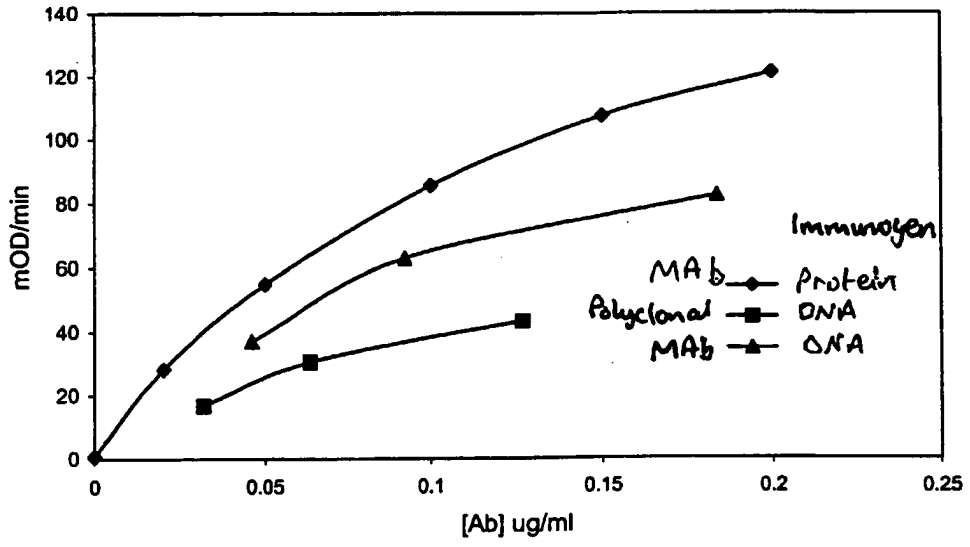

(57) Abstract: The invention uses the power of display selection methods to screen libraries of human immunoglobulin genes from nonhuman transgenic animals expressing human immunoglobulins. Such screening produces unlimited numbers of high affinity human antibodies to any target of interest.

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HUMAN ANTIBODIES

CROSS-REFERENCES TO RELATED APPLICATION

The present application derives priority from USSN, 60/157415, filed October 2, 1999 and 09/453,234, filed December 1, 1999, each of which is incorporated by reference in its entirety for all purposes.

BACKGROUND

Over recent years, it has become apparent that mouse antibodies are not ideal reagents for in vivo use due to induction of human anti-mouse responses in recipient patients. A number of solutions have been proposed including the production of chimeric and humanized antibodies (Queen et al., *Proc. Natl. Acad. Sci. USA* 86:10029-10033 (1989) and WO 90/07861, US 5,693,762, US 5,693,761, US 5,585,089, US 5,530,101 and Winter, US 5,225,539).

Human monoclonals antibodies are advantageous compared with those from mouse or other species, because, *inter alia*, they exhibit little or no immunogenicity in a human host. However, conventional technology for producing murine monoclonals cannot be applied unmodified to production of human antibodies for several reasons. First, mouse procedures typically involve sacrificing the mouse, a procedure that is obviously unacceptable to humans. Second, humans cannot be immunized with many types of antigens, including human antigens, due to the risk of inducing an undesired immune response. Third, forming immortalized derivatives of human B cells has proved more difficult than for mouse B cells

Early techniques for producing human antibodies met with only limited success. For example, immortalization of immunized human lymphocytes with Epstein-Barr virus, while successful in forming monoclonal-antibody secreting cultures, has often failed to produce cells having sufficiently long lifespans to provide a reliable source of the desired antibody. Kozbor et al. (1982), *Hybridoma* 1:323. In another approach, hybridomas generated by fusion of immunized human lymphoid cell lines with mouse myelomas, were found to exhibit chromosomal instability. Nowinski et al. (1980), *Science* 210:537; Lane et al. (1982), *J. Exp. Med.* 155:133 (1982).

Another approach has been described by Ostberg et al. (1983), *Hybridoma* 2:361-367 and Engelman et al., US Patent 4,634,666. This method entails

fusing a mouse myeloma cell with a nonimmunized human B-lymphocyte to form a xenogenic fusion cell. The fusion cell is then fused with an immunized human B-lymphocyte to produce a trioma cell. A number of human monoclonal antibodies to viral pathogens have been isolated using this approach.

A further approach has used the phage display technique to screen libraries of immunoglobulin genes obtained directly from human lymphatic cells from a naïve human. A basic concept of phage display methods is the establishment of a physical association between DNA encoding an antibody to be screened and the antibody chain. This physical association is provided by the phage particle, which displays an antibody as part of a capsid enclosing the phage genome which encodes the antibody. The establishment of a physical association between antibodies and their genetic material allows simultaneous mass screening of very large numbers of phage bearing different antibodies. Phage displaying an antibody with affinity to a target bind to the target and these phage are enriched by affinity screening to the target. The identity of antibodies displayed from these phage can be determined from their respective genomes. Using these methods an antibody identified as having a binding affinity for a desired target can then be synthesized in bulk by conventional means. Although the phage display method provides a powerful means of selection, the number of potential antibodies to be analyzed in a naïve human library is very large, about 10^{12}. Further, many of the antibodies in such a library are nonnaturally occurring combinations of heavy and light chain resulting from the random manner in which populations of these chains are combined when being cloned into the phage display vector. Such nonnaturally occurring combinations often lack capacity for strong binding. Thus, desired human antibodies with strong affinity for a human antibody are typically rare and consequently difficult to isolate from such libraries.

Human antibodies can also be produced from non-human transgenic mammals having transgenes encoding human immunoglobulin genes and having an inactivated endogenous immunoglobulin locus. The transgenic mammals resulting from this process are capable of functionally rearranging the immunoglobulin component sequences, and expressing a repertoire of antibodies of various isotypes encoded by human immunoglobulin genes, without expressing endogenous immunoglobulin genes. The production and properties of mammals having these properties are reported by, e.g., Lonberg et al., WO93/12227 (1993); US 5,877,397, US 5,874,299, US 5,814,318, US 5,789,650, US 5,770,429, US 5,661,016, US

5,633,425, US 5,625,126, US 5,569,825, US 5,545,806, *Nature* 148, 1547-1553 (1994), *Nature Biotechnology* 14, 826 (1996), Kucherlapati, WO 91/10741 (1991) (each of which is incorporated by reference in its entirety for all purposes). Antibodies are obtained by immunizing a transgenic nonhuman mammal, such as described by Lonberg or Kucherlapati, *supra,* with antigen Monoclonal antibodies are prepared by fusing B-cells from such mammals to suitable myeloma cell lines using conventional Kohler-Milstein technology.

SUMMARY OF THE INVENTION

The invention provides methods of producing a human antibody display library. Such methods entail providing a nonhuman transgenic animal whose genome comprises a plurality of human immunoglobulin genes that can be expressed to produce a plurality of human antibodies. A population of nucleic acids encoding human antibody chains is isolated from lymphatic cells of the nonhuman transgenic animal. The nucleic acids are then introduced into a display vector to provide a library of display packages, in which a library member comprises a nucleic acid encoding an antibody chain, and the antibody chain is displayed from the package.

In some methods, library members are contacted with a target. Library members displaying an antibody chain and binding partner (if present) with specific affinity for the target bind to the target, to produce a subpopulation of display packages. The resulting subpopulation of display packages typically comprises at least ten different display packages comprising at least ten nucleic acids encoding at least ten antibody chains. At least 50% of the nucleic acids typically encode human antibody chains, which with the binding partner (if present) show at least 10^8 M^{-1} affinity for the target and no library member constitutes more than 50% of the library.

Some methods entails a step of preenriching lymphatic cells before cloning antibody sequences. The subpopulation is enriched for lymphatic cells expressing an IgG antibody before the isolating step. The subpopulation can be prepared by contacting the isolated lymphatic cells with a reagent that binds to the Fc region of an IgG antibody. In addition, nucleic acids can be cloned from the lymphatic cells using a pair of primers one of which is specific for DNA encoding IgG heavy chains. In some methods, at least 90% of the human antibody chains cloned into a display vector have IgG isotype..

In some methods, nucleic acids having affinity for a target have a median of at least 2 somatic mutations per antibody chain encoded by the nucleic acids. In some methods, the nucleic acids having affinity for the target have a median of at least 5 somatic mutations per antibody chain encoded by the nucleic acids.

In some methods, the lymphatic cells are obtained from bone marrow.

In some methods, the lymphatic cells are from a nonhuman transgenic animal that has been immunized with an immunogen without developing a titer to the immunogen greater than ten fold of a negative control. In some methods, the lymphatic cells are from a nonhuman transgenic animal that has been immunized with an immunogen without developing a detectable titer against the immunogen.

In some methods, the display members are screened with a target is expressed on the surface of a cell. In some methods, the target is a protein within a phospholipid membrane or particle.

The invention also provides methods of producing a human Fab phage display library. Such methods entail providing a nonhuman transgenic animal whose genome comprises a plurality of human immunoglobulin genes that can be expressed to produced a plurality of human antibodies. Populations of nucleic acids respectively encoding human antibody heavy chains and human antibody light chains are isolated from lymphatic cells of the nonhuman transgenic animal. The populations are cloned into multiple copies of a phage display vector to produce a display library, in which a library member comprises a phage capable of displaying from its outersurface a fusion protein comprising a phage coat protein, a human antibody light chain or human antibody heavy chain. In at least some members, the human antibody heavy or light chain is complexed with a partner human antibody heavy or light chain, the complex forming a Fab fragment to be screened.

Library members are typically contacted with a target. Library members displaying a complex of a human heavy and light chain with specific affinity for the target bind to the target, to produce a subpopulation of display packages. The resulting subpopulation of display packages typically comprises at least ten different display packages comprising at least ten pairs of nucleic acids encoding at least ten pairs of heavy and light chains. Typically at least 50% of the pairs of nucleic acids encoding pairs of heavy and light chains forming complexes showing at least 10^8 M^{-1} affinity for the target and no library member constitutes more than 50% of the library.

In some methods, lymphatic cells re preenriched to prepare a subpopulation of lymphatic cells expressing an IgG antibody. In some methods, the subpopulation is prepared by contacting the isolated lymphatic cells with a reagent that binds to the Fc region of an IgG antibody. In some methods, DNA is isolated from lymphatic cells using a pair of primers one of which is specific for DNA encoding IgG heavy chains.

In some methods, pairs of nucleic acids encoding antibodies with specific affinity for the target have a median of at least 10 mutations in the nucleic acids encoding heavy chains and a median of at least two somatic mutations in the nucleic acids encoding light chains. In some methods, the pairs of nucleic acids have a median of at least 10 somatic mutations in the nucleic acids encoding the heavy chains and at least five somatic mutations in the nucleic acids encoding the light chains. In some methods, the pairs of nucleic acids have a median of at least ten somatic mutations in the nucleic acids encoding the heavy chains and a median of at least ten somatic mutations in the nucleic acids encoding the light chains.

In some methods, the lymphatic cells are obtained from bone marrow.

In some methods, the lymphatic cells are from a nonhuman transgenic mammal that has been immunized with an immunogen without developing a significant titer to the immunogen.

In some methods, the target is expressed on the surface of a cell.

In some methods at least 90% of the human antibody heavy chains encoded by a display vector have IgG isotype..

In some methods, the populations of nucleic acids respectively encode populations of human heavy and light chain variable regions, and the phage display vector encodes human heavy and light chain constant regions expressed in frame with human heavy and light chains inserted into the vector.

In some methods, the fusion protein encoded by the phage display vector further comprises a tag that is the same in different library members. Library members are screened for binding to a receptor with specific affinity for the tag.

In some methods, a mixed population of nucleic acids encoding human antibody heavy chains and human antibody light chains from the further subpopulation of library members is cloned into multiple copies of an expression vector to produce modified expression vectors.

The invention further provides libraries of at least ten different nucleic segments encoding human antibody chains. At least 50% of segments in the library encode human antibody chains showing at least 10^8 M^{-1} affinity for the same target and no library member constitutes more than 50% of the library. In some libraries, at least 90% of the pairs of different nucleic acid segments encode heavy and light chains that form complexes having at least 10^9 M^{-1} affinity of the target.

Some libraries comprise at least ten pairs of different nucleic acid segments, the members of a pair respectively encoding heavy and light human antibody chains, wherein at least 50% of the pairs encode heavy and light human antibody chains that form complexes showing specific affinity for the same target, and no pair of nucleic acid segments constitutes more than 50% of the library. Some libraries comprise at least 100 or 1000 pairs of different nucleic acid segments.

In some libraries, at least 50% of the pairs encode heavy and light chains that form complexes having affinity of at least 10^9 M^{-1} for the target. In some libraries, at least 50 or 90% of the pairs encode heavy and light chains that form complexes having affinity of at least 10^{10} M^{-1} for the target. In some libraries, at least the pairs of different nucleic acid segments encoding antibodies with specific affinity for the target have a median of at least 10 somatic mutations in the nucleic acids encoding the heavy chains and a median of at least 2 somatic mutations in the nucleic acids encoding the light chains. In some libraries, the pairs of different nucleic acid segments encoding antibodies with affinity for the target have a median of at least 10 somatic mutations in the nucleic acids encoding the heavy chains and a median of at least 10 somatic mutations in the nucleic acids encoding the light chains. In some libraries, at least 90% of pairs of different nucleic acids segments have a nucleic acid segment encoding a heavy chain of IgG isotype.

The invention further provides libraries of at least 1000 different nucleic segments encoding human antibody chains, wherein at least 90% of segments in the library encode human antibody chains for the same target and no library member constitutes more than 50% of the library, wherein each segment comprises subsequence(s) from a human VH and/or a human VL gene, and no more than 40 human VH genes and no more than 40 human VL genes are represented in the library.

The invention further provides a method of producing a human antibody display library in which an immunogen is introduced into a nonhuman transgenic animal whose genome comprises a plurality of human immunoglobulin

genes that can be expressed to produce a plurality of human antibodies. A population of nucleic acids encoding human antibody chains is isolated from lymphatic cells of the nonhuman transgenic animal, the nonhuman transgenic animal lacking a detectable titer or having a titer to the immunogen less than ten fold the background titer before immunization. A library of display packages is formed displaying the antibody chains, in which a library member comprises a nucleic acid encoding an antibody chain, and the antibody chain is displayed from the package. In some methods, the immunogen is a nucleic acid. In some methods, the nucleic acid encodes a membrane bound receptor.

The invention further provides a method of producing a human antibody display library in which a nonhuman transgenic animal whose genome comprises a plurality of human immunoglobulin genes that can be expressed to produce a plurality of human antibodies is provided. Lymphatic cells are obtained from the nonhuman mammal and the cells are enriched to produce a subpopulation or cells expressing antibodies of IgG isotype. Populations of nucleic acids encoding human heavy and light antibody chains are then isolated from the subpopulation. A library of display packages is formed displaying the human heavy and light antibody chains, in which a library member comprises nucleic acids encoding human antibody heavy and light chains, and a complex of the heavy and light chains is displayed from the library member. In some such methods, the nucleic acids encoding the human antibody heavy chains and the nucleic acids encoding the human antibody light chains both have a median of at least 5 somatic mutations per nucleic acid.

The invention further provides a method of producing a human antibody display library. Such methods entail introducing a nucleic acid encoding a protein immunogen into a nonhuman transgenic animal whose genome comprises a plurality of human immunoglobulin genes that can be expressed to produce a plurality of human antibodies. A population of nucleic acids encoding human antibody chains is isolated from lymphatic cells of the nonhuman transgenic animal. A library of display packages displaying the antibody chains is formed in which a library member comprises a nucleic acid encoding an antibody chain, and the antibody chain is displayed from the package. In some methods, the immunogen is a natural protein. In some the nucleic acid encodes a membrane bound protein or an EST. In some such methods, library members are contacted with a target so that library members displaying an antibody chain and binding partner (if present) with specific affinity for

the target bind to the target, to produce a subpopulation of display packages. This screening can result in a subpopulation of display packages comprises at least ten different display packages comprising at least ten nucleic acids encoding at least ten antibody chains, and at least 50% of the nucleic acids encode human antibody chains, which in combination with a binding partner (if present) show at least 10^{10} M^{-1} affinity for the target and no library member constitutes more than 50% of the library

The invention further provides a method of preparing a population of antibodies. Such methods employ a first library of display packages displaying antibody chains, in which a library member comprises a nucleic acid encoding an antibody chain, and the antibody chain is displayed from the package. Such a library is screened for binding to a target to isolate a first population of display packages displaying antibody chains that specifically bind to the target. One then screens a second library of similar display packages displaying antibody chains for binding to the target, the screening being conducted in the presence of antibodies displayed from the first population of display packages to generate a second population of display packages displaying antibody chains that specifically bind to the target. The antibody chains in the second population of chains and the antibody chains in the first population of chain have different epitope binding profiles in the target.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1: shows a vector obtained from Ixsys, Inc. and described in Huse, WO 92/06204, which provides the starting material for producing phage display vectors. The following abbreviations are used:

A. Nonessential DNA sequence later deleted.

B. Lac promoter and ribosome binding site.

C. Pectate lyase signal sequence.

D. Kappa chain variable region.

E. Kappa chain constant region.

F. DNA sequence separating kappa and heavy chain, includes ribosome binding site for heavy chain.

G. Alkaline phosphatase signal sequence.

H. Heavy chain variable region.

I. Heavy chain constant region including 5 amino acids of the hinge region.

J. Decapeptide DNA sequence.

K. Pseudo gene VIII sequence with amber stop codon at 5' end.

L. Nonessential DNA sequence that was later deleted.

Fig. 2: Oligonucleotides used in vector construction.

Fig. 3: Map of the vector pBRncoH3.

Fig. 4: Insertion of araC into pBR-based vector (Fig. 4A) and the resulting vector pBRnco (Fig. 4B).

Fig. 5: Subcloning of a DNA segment encoding a Fab by T4 exonuclease digestion.

Fig. 6 Targeted insertion of a neo cassette into the SmaI site of the mu1 exon. A. Schematic diagram of the genomic structure of the mu locus. The filled boxes represent the mu exons. B. Schematic diagram of the CmuD targeting vector. The dotted lines denote those genomic mu sequences included in the construct. Plasmid sequences are not shown. C. Schematic diagram of the targeted mu locus in which the neo cassette has been inserted into mu1. The box at the right shows those RFLP's diagnostic of homologous recombination between the targeting construct and the mus locus. The FGLP's were detected by Southern blot hybridization using probe A, the 915 SaI fragment shown in C.

Fig. 7 Nongermline encoded nucleotides in heavy and light chain V genes. Heavy chain V genes were found to be heavily somatically mutated. Light chain V genes comprised fewer non-germline encoded nucleotides.

Fig. 8: ELISA for monoclonal antibodies to troponin derived from HuMAb mice showing zero titer.

Fig. 9: ELISA for poly and monoclonal antibodies to IL-8 prepared by immunization with DNA compared with a monoclonal prepared by immunization with purified IL-8.

Fig. 10: ELISA of polyclonal antibodies to oxidized troponin prepared from bone marrow of HuMAb mice.

DEFINITIONS

Specific binding between an antibody or other binding agent and an antigen means a binding affinity of at least 10^6 M^{-1}. Preferred binding agents bind with affinities of at least about 10^7 M^{-1}, and preferably 10^8 M^{-1} to 10^9 M^{-1},10^{10} M^{-1}, 10^{11} M^{-1}, 10^{12} M^{-1}or 10^{13} M^{-1}. The term epitope means an antigenic determinant

capable of specific binding to an antibody. Epitopes usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and usually have specific three dimensional structural characteristics, as well as specific charge characteristics. Conformational and nonconformational epitopes are distinguished in that the binding to the former but not the latter is lost in the presence of denaturing solvents.

The basic antibody structural unit is known to comprise a tetramer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one "light" (about 25 kDa) and one "heavy" chain (about 50-70 Kda). The amino-terminal portion of each chain includes a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The carboxyl-terminal portion of each chain defines a constant region primarily responsible for effector function.

Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, and define the antibody's isotype as IgG, IgM, IgA, IgD and IgE, respectively. Within light and heavy chains, the variable and constant regions are joined by a "J" region of about 12 or more amino acids, with the heavy chain also including a "D" region of about 10 more amino acids. (See generally, Fundamental Immunology (Paul, W., ed., 2nd ed. Raven Press, N.Y., 1989, 4th edition (1999), Paul William E., ed. Raven Press, N.Y.,(incorporated by reference in its entirety for all purposes). The genes encoding variable regions of heavy and light immunoglobulin chains are referred to as V_H and V_L respectively. Although the amino acid sequence of an immunoglobulin chain is not exactly the same as would be predicted from the V_H or V_L gene that encoded it due to somatic mutations (see Fig. 7), there is sufficient similarity between predicted and actual sequences of immunoglobulins that the actual sequence is characteristic and allows recognition of a corresponding V_H or V_L gene. The term constant region is used to refer to both full-length natural constant regions and segments thereof, such as C_H1, hinge, C_H2 and C_H3 or fragments thereof. Typically, segments of light and heavy chain constant regions in antibodies have sufficient length to contribute to interchain bonding between heavy and light chain.

The variable regions of each light/heavy chain pair form the antibody binding site. Thus, an intact antibody has two binding sites. Except in bifunctional or bispecific antibodies, the two binding sites are the same. The chains all exhibit the

same general structure of four relatively conserved framework regions (FR) joined by three hypervariable regions, also called complementarily determining regions or CDRs. The CDRs from the two chains of each pair are aligned by the framework regions, enabling binding to a specific epitope. CDR and FR residues are delineated according to the standard sequence definition of Kabat, *et al., supra.* An alternative structural definition has been proposed by Chothia, *et al., J. Mol. Biol.* **196**:901-917 (1987); *Nature* **342**:878-883 (1989); and *J. Mol. Biol.* **186**:651-663 (1989).

The term antibody is used to mean whole antibodies and binding fragments thereof. Binding fragments include single chain fragments, Fv fragments and Fab fragments The term Fab fragment is sometimes used in the art to mean the binding fragment resulting from papain cleavage of an intact antibody. The terms Fab' and F(ab')2 are sometimes used in the art to refer to binding fragments of intact antibodies generated by pepsin cleavage. Here, Fab is used to refer generically to double chain binding fragments of intact antibodies having at least substantially complete light and heavy chain variable domains sufficient for antigen-specific bindings, and parts of the light and heavy chain constant regions sufficient to maintain association of the light and heavy chains. Usually, Fab fragments are formed by complexing a full-length or substantially full-length light chain with a heavy chain comprising the variable domain and at least the C_H1 domain of the constant region.

An isolated species or population of species means an object species (*e.g.*, binding polypeptides of the invention) that is the predominant species present (*i.e.*, on a molar basis it is more abundant than other species in the composition). Preferably, an isolated species comprises at least about 50, 80 or 90 percent (on a molar basis) of all macromolecular species present. Most preferably, the object species is purified to essential homogeneity (contaminant species cannot be detected in the composition by conventional detection methods). A target is any molecule for which it is desired to isolate partners with specific binding affinity for the target.

Targets of interest include antibodies, including anti-idiotypic antibodies and autoantibodies present in autoimmune diseases, such as diabetes, multiple sclerosis and rheumatoid arthritis. Other targets of interest are growth factor receptors (*e.g.*, FGFR, PDGFR, EFG, NGFR, and VEGF) and their ligands. Other targets are G-protein receptors and include substance K receptor, the angiotensin receptor, the - and -adrenergic receptors, the serotonin receptors, and PAF receptor. *See, e.g.,* Gilman, *Ann. Rev. Biochem.* **56**:625-649 (1987). Other targets include ion

channels (*e.g.*, calcium, sodium, potassium channels), muscarinic receptors, acetylcholine receptors, GABA receptors, glutamate receptors, and dopamine receptors (*see* Harpold, 5,401,629 and US 5,436,128). Other targets are adhesion proteins such as integrins, selectins, and immunoglobulin superfamily members (*see* Springer, *Nature* **346**:425-433 (1990). Osborn, *Cell* **62**:3 (1990); Hynes, *Cell* **69**:11 (1992)). Other targets are cytokines, such as interleukins IL-1 through IL-13, tumor necrosis factors & , interferons , and , tumor growth factor Beta (TGF-), colony stimulating factor (CSF) and granulocyte monocyte colony stimulating factor (GM-CSF). *See* Human Cytokines: Handbook for Basic & Clinical Research (Aggrawal *et al.* eds., Blackwell Scientific, Boston, MA 1991). Other targets are hormones, enzymes, and intracellular and intercellular messengers, such as, adenyl cyclase, guanyl cyclase, and phospholipase C. Drugs are also targets of interest. Target molecules can be human, mammalian or bacterial. Other targets are antigens, such as proteins, glycoproteins and carbohydrates from microbial pathogens, both viral and bacterial, and tumors. Still other targets are described in US 4,366,241. Some agents screened by the target merely bind to a target. Other agents agonize or antagonize the target.

Display library members having full-length polypeptide coding sequences have coding sequences the same length as that of the coding sequences originally inserted into a display vector before propagation of the vector.

The term phage is used to refer to both phage containing infective genomes and phage containing defective genomes that can be packaged only with a helper phage. Such phage are sometimes referred to as phagemids.

The term "human antibody" includes antibodies having variable and constant regions (if present) derived from human germline immunoglobulin sequences. Human antibodies of the invention can include amino acid residues not encoded by human germline immunoglobulin sequences (*e.g.*, mutations introduced by random or site-specific mutagenesis *in vitro* or by somatic mutation *in vivo*). However, the term "human antibody" does not include antibodies in which CDR sequences derived from the germline of another mammalian species, such as a mouse, have been grafted onto human framework sequences (i.e., humanized antibodies).

A rearranged heavy chain or light chain immunoglobulin locus has a V segment positioned immediately adjacent to a D-J or J segment in a conformation encoding essentially a complete V_H or V_L domain, respectively. A rearranged

immunoglobulin gene locus can be identified by comparison to germline DNA; the rearranged locus having at least one recombined heptamer/nonamer homology element. Conversely, an unrearranged or germline configuration refers to a configuration in which the V segment is not recombined so as to be immediately adjacent to a D or J segment.

"Isotype switching" refers to the phenomenon by which the class, or isotype, of an antibody changes from one Ig class to one of the other Ig classes.

"Nonswitched isotype" refers to the isotypic class of heavy chain that is produced when no isotype switching has taken place; the C_H gene encoding the nonswitched isotype is typically the first CH gene immediately downstream from the functionally rearranged VDJ gene. Isotype switching has been classified as classical or non-classical isotype switching. Classical isotype switching occurs by recombination events that involve at least one switch sequence region in the transgene. Non-classical isotype switching may occur by, for example, homologous recombination between human σ_μ and human $\sum_\mu$ (δ-associated deletion). Alternative non-classical switching mechanisms, such as intertransgene and/or interchromosomal recombination, among others, may occur and effectuate isotype switching.

The term "switch sequence" refers to those DNA sequences responsible for switch recombination. A "switch donor" sequence, typically a μ switch region, are 5' (*i.e.*, upstream) of the construct region to be deleted during the switch recombination. The "switch acceptor" region are between the construct region to be deleted and the replacement constant region (*e.g.*, γ, ε, etc.). As there is no specific site where recombination always occurs, the final gene sequence is not typically predictable from the construct.

Somatic mutation and affinity maturation of antibody genes allows for the evolutionary selection of variable regions of antibodies based on binding affinity. However, this process differs from evolutionary natural selection of individuals from sexually reproducing species because there is no mechanism to allow for the combination of separately selected beneficial mutations. The absence of recombination between individual B cells requires that beneficial mutations be selected for sequentially. Theoretically, combinatorial libraries allow for such combinations (at least in the case where the two mutations are on heavy and light chains respectively). However, combinatorial libraries derived from natural sources

include such a wide diversity of different heavy/light chain pairs that the majority of the clones are not derived from the same B cell bone marrow precursor cell. Such pairings are less likely to form stable antibody molecules that recognize the target antigen.

DETAILED DESCRIPTION

I. General

The present invention uses display methods to screen libraries of antibodies originally expressed in nonhuman transgenic animals to produce populations of human antibodies having unexpected characteristics. These characteristics include unusually high binding affinities (e.g., pM dissociation constants in some instances), virtually unlimited numbers of such antibodies, and a high degree of enrichment for such antibodies in the population. The methods of the invention typically work by immunizing a nonhuman transgenic animal having human immunoglobulin genes. The animal expresses a diverse range of human antibodies that bind to the antigen. Nucleic acids encoding the antibody chain components of such antibodies are then cloned from the animal into a display vector. Typically, separate populations of nucleic acids encoding heavy and light chain sequences are cloned, and the separate populations then recombined on insertion into the vector, such that any given copy of the vector receives a random combination of a heavy and light chains. The vector is designed to express antibody chains so that they can be assembled and displayed on the outersurface of a display package containing the vector. For example, antibody chains can be expressed as fusion proteins with a phage coat protein from the outersurface of the phage. Thereafter, display packages can be screened for display of antibodies binding to a target.

In some methods, display packages are subject to a prescreening step. In such methods, the display package encode a tag expressed as a fusion protein with an antibody chain displayed from the package. Display packages are prescreened for binding to a receptor to the tag. The prescreening step serves to enrich for display packages displaying multiple copies of an antibody chain linked to the tag. It is believed that it is this subset of display packages that binds to target in the subsequent screening step to a target. After prescreening with receptor (if any) and screening with target, display packages binding to the target are isolated, and optionally, subject to further rounds screening to target, with each such round optionally being preceded

by prescreening to receptor. By including one or more rounds of prescreening with a tag, the extent of enrichment can increase to approaching or even beyond 99% in contrast with conventional procedures in which the extent of enrichment typically plateaus after a few rounds of screening at around 10-20%. Display packages are typically amplified between rounds of screening to target but not between prescreening and screening steps. After one or a few rounds of screening to target, the remaining display packages are highly enriched for high affinity binders to the target. Furthermore, the conditions of screening can be controlled to select antibodies having affinity in excess of a chosen threshold.

In some methods, nucleic acids encoding human antibody chains are subcloned en masse from display vectors surviving selection to an expression vector. Typically, a nucleic acid encoding both heavy and light chains of an antibody displayed from a display package is subcloned to an expression vector thereby preserving the same combinations of heavy and light chains in expression vectors as were present in the display packages surviving selection. The expression vector can be designed to express inserted antibody chains as Fab fragments, intact antibodies or other fragments. Cloning en masse of nucleic acids encoding antibody chains into an expression vector and subsequent expression of the vector in host cells results in a polyclonal population of intact human antibodies or fragments thereof. Such a population contains a diverse mixture of different antibody types, the majority of which types show very high affinity for the same target, albeit usually to different epitopes within the target.

It is believed that the success of the invention in providing virtually unlimited numbers of unusually high affinity human antibodies to any desired target (see Example 21) results in part from reducing the total number of combinations of heavy and light chains that might form by random combination of the respective repertoires of these chains in the human repertoire. Display methods provide a means for screening vast numbers of antibodies for desired properties. However, the random association of light and heavy chains that occurs on cloning into a display vector results in unnatural combinations of heavy and light chains that may be nonfunctional. When heavy and light chains are cloned from a natural human, the number of permutations of heavy and light chains is very high, and probably a very large proportion of these are nonnaturally occurring and not capable of high affinity binding. Thus, high affinity antibodies constitute a very small proportion of such

libraries and are difficult to isolate. Nonhuman transgenic animals with human immunoglobulin genes typically do not include the full complement of human immunoglobulin genes present in a natural human. It is believed that the more limited complement of human immunoglobulin genes present in such animals results in a reduced proportion of unnatural random permutations of heavy and light chains incapable of high affinity binding. Thus, when the vast power of display selection is applied free of the burden of very large numbers of unnatural combinations inherent in previous methods, indefinitely large numbers of human immunoglobulins having very high affinities result.

The enrichment for productive combinations of heavy and light chains afforded by use of transgenic animals with less than a full complement of human genes can be supplemented or substituted by preenrichment for lymphatic cells expressing IgG heavy chains and their binding partners. IgG heavy chains typically show strongest affinity for a target antigen. By enriching for cells expressing such chains and their natural light chain partners before random combination of nucleic acids attendant to introduction into a display vector, one produces a display library containing a higher proportion of heavy and light chain combinations with potential for tight binding to a target. Although random association between heavy and light chains still produces nonnaturally occurring combinations of heavy and light chains, these combinations are formed from component chains of the tightest binding natural antibodies, are more likely therefore to be themselves tight binding antibodies. Enrichment for IgG increases the proportion of IgG antibodies in selected display libraries, and increases the median number of somatic mutations in nucleic acids encoding antibody chains in selected display libraries. By preenriching for both IgG chains and their binding partners, the median number of somatic mutations increases in both heavy and light chains. The increase is particular notable for the light chain because no this chain is not subjected to further enrichment at the PCR stage by use of iostype specific primers. Thus, in methods employing IgG enrichment of B cells, the number of somatic mutations per light chain approaches or equals that per heavy chain. Although the mechanisms discussed above are believed to explain in part the results achieved using the invention, practice of the invention is not dependent on the correctness of this belief.

II. Production of Antibodies in Transgenic Animals with Human Immune Systems

A. Transgenic Animals

The transgenic animals used in the invention bear a heterologous human immune system and typically a knocked out endogenous immune systems. Mice are a preferred species of nonhuman animal. Such transgenic mice sometimes referred to as HuMAb mice contain a human immunoglobulin gene miniloci that encodes unrearranged human heavy (μ and γ) and κ light chain immunoglobulin sequences, together with targeted mutations that inactivate the endogenous μ and κ chain loci (Lonberg *et al.* (1994) *Nature* 368(6474): 856-859 and US patent 5,770,429). Accordingly, the mice exhibit reduced expression of mouse IgM or κ, and in response to immunization, the introduced human heavy and light chain transgenes undergo class switching and somatic mutation to generate high affinity human IgGκ monoclonal (Lonberg *et al.* (1994), *supra*; reviewed in Lonberg, N. (1994) H*andbook of Experimental Pharmacology* 113:49-101; Lonberg and Huszar, . (1995) *Intern. Rev. Immunol.* Vol. 13: 65-93, and Harding. and Lonberg (1995) *Ann. N.Y. Acad. Sci* 764:536-546); Taylor, L. *et al.* (1992) *Nucleic Acids Research* 20:6287-6295; Chen, J. *et al.* (1993) *International Immunology* 5: 647-656; Tuaillon *et al.* (1993) *Proc. Natl. Acad. Sci USA* 90:3720-3724; Choi *et al.* (1993) *Nature Genetics* 4:117-123; Chen, J. *et al.* (1993) EMBO J. 12: 821-830; Tuaillon *et al.* (1994) *J. Immunol.* 152:2912-2920; Lonberg *et al.*, (1994) *Nature* 368(6474): 856-859; Lonberg, N. (1994) *Handbook of Experimental Pharmacology* 113:49-101; Taylor, L. *et al.* (1994) *International Immunology* 6: 579-591; Lonberg, N. and Huszar, D. (1995) Intern. Rev. Immunol. Vol. 13: 65-93; Harding, F. and Lonberg, N. (1995) *Ann. N.Y. Acad. Sci* 764:536-546; Fishwild, D. *et al.* (1996) *Nature Biotechnology* 14: 845-851; U.S. Patent Nos. 5,625,126 and 5,770,429 US 5,545,807, US 5,939,598, WO 98/24884, WO 94/25585, WO 93/1227, WO 92/22645, WO 92/03918, the disclosures of all of which are hereby incorporated by reference in their entity.

Some transgenic non-human animals are capable of producing multiple isotypes of human monoclonal antibodies to an antigen (*e.g.*, IgG, IgA and/or IgE) by undergoing V-D-J recombination and isotype switching. Isotype switching may occur by, *e.g.*, classical or non-classical isotype switching.

Transgenic non-human animal are designed so that human immunoglobulin transgenes contained within the transgenic animal function correctly throughout the pathway of B-cell development. In some mice, correct function of a heterologous heavy chain transgene includes isotype switching. Accordingly, the transgenes of the invention are constructed so as to produce isotype switching and one or more of the following: (1) high level and cell-type specific expression, (2) functional gene rearrangement, (3) activation of and response to allelic exclusion, (4) expression of a sufficient primary repertoire, (5) signal transduction, (6) somatic hypermutation, and (7) domination of the transgene antibody locus during the immune response.

In transgenic animals in which the endogenous immunoglobulin loci of the transgenic animals are functionally disrupted, the transgene need not activate allelic exclusion. Further, in transgenic animals in which the transgene comprises a functionally rearranged heavy and/or light chain immunoglobulin gene, the second criteria of functional gene rearrangement is unnecessary, at least for transgenes that are already rearranged.

Some transgenic non-human animals used to generate the human monoclonal antibodies contain rearranged, unrearranged or a combination of rearranged and unrearranged heterologous immunoglobulin heavy and light chain transgenes in the germline of the transgenic animal. In addition, the heavy chain transgene can contain functional isotype switch sequences, which are capable of supporting isotype switching of a heterologous transgene encoding multiple CH genes in the B-cells of the transgenic animal. Such switch sequences can be those which occur naturally in the germline immunoglobulin locus from the species that serves as the source of the transgene CH genes, or such switch sequences can be derived from those which occur in the species that is to receive the transgene construct (the transgenic animal). For example, a human transgene construct that is used to produce a transgenic mouse may produce a higher frequency of isotype switching events if it incorporates switch sequences similar to those that occur naturally in the mouse heavy chain locus, as presumably the mouse switch sequences are optimized to function with the mouse switch recombinase enzyme system, whereas the human switch sequences are not. Switch sequences can be isolated and cloned by conventional cloning methods, or can be synthesized de novo from overlapping synthetic oligonucleotides designed on the basis of published sequence information relating to

immunoglobulin switch region sequences (Mills *et al.*, *Nucl. Acids Res.* 15:7305-7316 (1991); Sideras *et al.*, *Intl. Immunol.* 1:631-642 (1989) incorporated by reference). Typically, functionally rearranged heterologous heavy and light chain immunoglobulin transgenes are found in a significant fraction of the B-cells of the above transgenic animal (at least 10 percent).

The transgenes used to generate the transgenic animals of the invention include a heavy chain transgene comprising DNA encoding at least one variable gene segment, one diversity gene segment, one joining gene segment and at least one constant region gene segment. The immunoglobulin light chain transgene comprises DNA encoding at least one variable gene segment, one joining gene segment and at least one constant region gene segment. The gene segments encoding the light and heavy chain gene segments are heterologous to the transgenic non-human animal in that they are derived from, or correspond to, DNA encoding immunoglobulin heavy and light chain gene segments from a species other than the transgenic non-human animal., typically the human species.

Typically transgenes are constructed so that the individual gene segments are unrearranged, *i.e.*, not rearranged so as to encode a functional immunoglobulin light or heavy chain. Such unrearranged transgenes support recombination of the V, D, and J gene segments (functional rearrangement) and preferably support incorporation of all or a portion of a D region gene segment in the resultant rearranged immunoglobulin heavy chain within the transgenic non-human animal when exposed to antigen. Such transgenes typically comprise a substantial portion of the C, D, and J segments as well as a subset of the V gene segments.

In such transgene constructs, the various regulatory sequences, *e.g.* promoters, enhancers, class switch regions, splice-donor and splice-acceptor sequences for RNA processing, recombination signals and the like, comprise corresponding sequences derived from the heterologous DNA. Such regulatory sequences can be incorporated into the transgene from the same or a related species of the non-human animal used in the invention. For example, human immunoglobulin gene segments can be combined in a transgene with a rodent immunoglobulin enhancer sequence for use in a transgenic mouse. Alternatively, synthetic regulatory sequences can be incorporated into the transgene, wherein such synthetic regulatory sequences are not homologous to a functional DNA sequence that is known to occur naturally in the genomes of mammals. Synthetic regulatory sequences are designed

according to consensus rules, such as, for example, those specifying the permissible sequences of a splice-acceptor site or a promoter/enhancer motif. The transgene can comprise a minilocus.

Some transgenic animals used to generate human antibodies contain at least one, typically 2-10, and sometimes 25-50 or more copies of the transgene described in Example 37 of US patent 5,770,429, or the transgene described in Example 24 (*e.g.*, HCo12), at least one copy of a light chain transgene described in Examples 38 of US patent 5,770,429, two copies of the Cmu deletion described in Example 23, and two copies of the Jkappa deletion described in Example 9 of US patent 5,770,429, each incorporated by reference in its entirety for all purposes.

Some transgenic animals exhibit immunoglobulin production with a significant repertoire. Thus, for example, animals in which the endogenous Ig genes have been inactivated, the total immunoglobulin levels range from about 0.1 to 10 mg/ml of serum, preferably 0.5 to 5 mg/ml. The immunoglobulins expressed by the transgenic mice typically recognize about one-half or more of highly antigenic proteins, *e.g.*, staphylococcus protein A.

The transgenic nonhuman animals can be immunized with a purified or enriched preparation of antigen and/or cells expressing antigen. The animals produce B cells that undergo class-switching via intratransgene switch recombination (*cis*-switching) and express immunoglobulins reactive with the antigen with which they are immunized. The immunoglobulins can be human sequence antibodies, in which the heavy and light chain polypeptides are encoded by human transgene sequences, which can include sequences derived by somatic mutation and V region recombinatorial joints, as well as germline-encoded sequences. These human sequence immunoglobulins can be referred to as being substantially identical to a polypeptide sequence encoded by a human V_L or V_H gene segment and a human JL or JL segment, even though other non-germline sequences may be present as a result of somatic mutation and differential V-J and V-D-J recombination joints. With respect to such human sequence antibodies, the variable regions of each chain are typically at least 80 percent encoded by human germline V, J, and, in the case of heavy chains, D, gene segments; frequently at least 85 percent of the variable regions are encoded by human germline sequences present on the transgene; often 90 or 95 percent or more of the variable region sequences are encoded by human germline sequences present on the transgene. However, since non-germline sequences are introduced by somatic

mutation and VJ and VDJ joining, the human sequence antibodies frequently have some variable region sequences (and less frequently constant region sequences) which are not encoded by human V, D, or J gene segments as found in the human transgene(s) in the germline of the mice. Typically, such non-germline sequences (or individual nucleotide positions) cluster in or near CDRs, or in regions where somatic mutations are known to cluster.

The human sequence antibodies which bind to the predetermined antigen can result from isotype switching, such that human antibodies comprising a human sequence γ chain (such as γl, γ2, γ3, or γ4) and a human sequence light chain (such as kappa or lambda) are produced. Such isotype-switched human sequence antibodies often contain one or more somatic mutation(s), typically in the variable region and often in or within about 10 residues of a CDR) as a result of affinity maturation and selection of B cells by antigen, particularly subsequent to secondary (or subsequent) antigen challenge. Fig. 7 shows the frequency of somatic mutations in various immunoglobulins of the invention (without the benefit of enrichment for IgG B cells before cloning nucleic acids encoding antibody chains)..

B. Immunization

1. Immunization with antigen

HuMAb transgenic animals can be immunized intraperitoneally (IP) with antigen in complete Freund's adjuvant, followed by IP immunizations with antigen in incomplete Freund's adjuvant every two weeks or month for a few months. Adjuvants other than Freund's are also effective. In addition, whole cells in the absence of adjuvant are found to be highly immunogenic. The immune response can be monitored over the course of the immunization protocol with plasma samples being obtained by retroorbital bleeds. Mice can be boosted intravenously with antigen 3 days before sacrifice and removal of the spleen.

In some methods, antibody libraries can be generated from immunized transgenic animals notwithstanding the absence of a significant titer to the antigen in serum from the animal. It is believed that such animals express mRNA without secreting significant amounts of antibody encoded by the mRNA. mRNA from such cells can thus be converted to cDNA and cloned into a display vector notwithstanding the absence of a detectable titer in the animal from which the mRNA was obtained.

The insight that an antibody titer is not required shortens the number of immunizations and period following the immunization that would be required according to conventional wisdom whereby the number of tight binding antibodies correlates with the extent of immune response. Accordingly, one or two immunizations with antigen, and a total period following the first immunization of one or two weeks are often sufficient for mRNA to be expressed and give rise to satisfactory antibody libraries by the display screening procedures described below. Additionally, the insight that antibody titer is not required extends the range of antigens that can be used to generate antibodies. Antigens that might previously have been rejected as immunogens through lack of measurable antibody titer in serum can now be used to produce mRNA libraries, and thus antibodies libraries according to the present methods.

In some methods of the invention, nucleic acids encoding mRNAs are harvested from transgenic animals immunized with an antigen but lacking any detectable titer. This means that the immune response of sera to the antigen is not significantly different (i.e., within experimental error) of a negative control. If serum is titered by binding to antigen immobilized to a solid phase, a suitable negative control is the solid phase without the antigen. Alternatively, serum from the animal before immunization can be used as a negative control. In some methods, nucleic acids are harvested from a transgenic animal showing a titer that is above the background level of a negative control but not to an extent that would by conventional wisdom be considered sufficient for production of tight binding antibodies. For example, the titer can be 2-fold, 5-fold, 10-fold, 50 fold or 100-fold above a negative control.

2. Immunization with DNA

In some methods, DNA is used as an immunogen. It is believed that the DNA is transcribed and translated in situ, and the translation product generates an immune response. Although there have been a few previous attempt at DNA immunization reported in the literature (see WO 99/28471, Chowdhury et al., *PNAS* 95, 669-674 (1998) and *J. Immunol. Methods* 231, 83-91 (1999)), this technique has been little used due to the lack of detectable titer generally observed, and the perception that such a strong titer is necessary to prepare antibodies. The present invention shows that mRNA encoding antibodies specific to the antigen encoded by the DNA is routinely produced in B-cells of such animals notwithstanding a lack of

detectable titer. The DNA can be cloned and used to produce antibody libraries according to the methods of the invention. Example 36 shows that a population of antibodies having very tight binding affinities of the order of 10^{12} M^{-1} affinity can be produced notwithstanding lack of detectable titer to the antigen in the transgenic animal from which mRNA was harvested.

Use of DNA as an immunogen has a number of advantages, particularly for generating an immune response to antigens that are difficult to purify, are available only in small amounts, if at all, or which lose their secondary structure on purification. Use of DNA as an immunogen is also useful for producing antibodies to proteins that have not yet been isolated or characterized, for example, the expression products of expressed sequence tags (ESTs). Because DNA immunization can produce antigens in a more native format than immunization with the antigen per se tighter binding antibodies can also be routinely produced. For example, affinities of at least 10^{10}, 10^{11}, 10^{12} or 10^{13} M^{-1} are possible. DNA immunization can be performed with or without an adjuvant. The adjuvant, if present, can be one that is typically used with a protein antigen, such as complete or incomplete Freund's adjuvant, or SDS, or it can be an adjuvant that is specifically chosen to associate with DNA, such as the positively charged detergent CTAB. The DNA to be used as an immunogen is typically operably linked to a promoter and other regulatory sequences required for its expression and translation. Optionally, the DNA is present as a component of a vector. In some instances, the vector encodes proinflammatory cytokines to attract immune cells to the site of injection. In some instances, the DNA encodes a fusion protein, comprising an antigenic component to which antibodies are desired and a T-cell antigen, such as tetanus toxoid, or other adjuvant such as C3d (see Dempsey et al., *Science* 271, 348-50 (1996)). The DNA can encode a full length protein or a desired epitopic fragment thereof. Typically, the DNA encodes a protein other than a random peptide, for example, a random peptide showing no more than chance resemblance to a natural protein. In some methods, the DNA encodes a natural protein, and in some methods, the natural protein is a human protein. Natural proteins include naturally occurring allelic variants.

C. Harvesting B cells

In conventional methods of antibody production using cell fusion, the spleen is usually used as a source of B-cells because B-cells from this source can readily be activated and thereby rendered amenable to fusion. In the present methods, in which no fusion is required, B-cells can be harvested from any tissue source irrespective whether the B-cells from that source are subjectable to activation and cell fusion. Sources of B-cells other than the spleen can be advantageous in some circumstances. For example B-cells from the bone marrow contain potentially interesting populations of B cells that are refractory to this activation. The bone marrow is a source of circulating high affinity antibodies long after germinal center formation of secondary repertoire B cells (Slifka et al. 1995, *J. Virol.* 69, 1895-1902; Takahashi et al, 1998, *J. Exp. Med.* 187, 885-895). Fusion of bone marrow cells does not efficiently access the memory B and plasma cells that encode these high affinity antibodies. Display systems provide a method for recovering the V region sequences of these useful, but otherwise unavailable antibodies. Use of bone marrow as a source of B-cells can also be advantageous in that bone marrow contains a smaller number of B-cell types and random combination of heavy and light chains from this reduced repertoire can lead to more productive combinations.

D. Enrichment for B-cells

In some methods, B-cells from spleen, bone marrow or other tissue source are subject to an enrichment procedure to select a subpopulation of cells that is enriched for heavy chains of IgG isotype and their natural binding partners. This subpopulation constitutes a secondary repertoire of B cells in which both heavy and light antibody chains have been subject to extensive affinity maturation. Enrichment can be accomplished by antibody mediated panning using anti-IgG antibodies to positively select for secondary repertoire B cells; or using anti-IgM and/or anti-IgD antibodies to negatively select against primary repertoire B cells. Negative selection can also be carried out using antibody mediated compliment lysis (Mishell and Shiigi, *Selected Methods in Cellular Immunology*, W.H. Freeman and Co., New York). Optionally, negative selection can be followed by addition of RNAse-free DNAse to the compliment lysis reaction mixture to prevent bystander killing and cross contamination by primary repertoire DNA released from lysed cells. Flow cytometric sorting can also be used to separate IgG positive B cells from IgM/IgD positive B

cells using fluorescently tagged antibodies. These methods select for B-cells bearing IgG heavy chains and their natural partner light chains, both of which have a higher median number of somatic mutations per cell that antibody chains from the total B-cell repertoire.

5

E. Cloning Nucleic Acids Encoding Antibodies from B cells

Nucleic acids encoding at least the variable regions of heavy and light chains can be cloned from either immunized or naïve transgenic animals. Nucleic acids can be cloned as genomic or cDNA from lymphatic cells of such animals. No
10 immortalization of such cells is required prior to cloning of immunoglobulin sequences. Usually, mRNA is isolated and amplified by reverse transcription with polydT primers. The cDNA is then amplified using primers to conserved regions of human immunoglobulins. The libraries can be easily enriched for non-mu isotypes using a 3' primer specific for non-mu sequences (e.g., IgG) Typically, the amplified
15 population of light chains comprises at least 100, 1000, 10,000, 100,000 or 1,000,000 different light chains. Likewise, the amplified population of heavy chains comprises at least 100, 1000, 10,000, 100,000 or 1,000,000 different heavy chains. Using IgG primers, typically at least 90, 95 or 99% of amplified heavy chains are of IgG isotype. If B-cell enrichment is performed, typically, at least 50%, 60%, 75%, 90%, 95% or
20 99% of light chains are the natural partners of IgG heavy chains.

III. Display Libraries

A. Display Packages

A display package, sometimes referred to as a replicable genetic
25 package, is a screenable unit comprising a polypeptide to be screened linked to a nucleic acid encoding the polypeptide. The nucleic acid should be replicable either in vivo (e.g., as a vector) or in vitro (e.g., by PCR, transcription and translation). In vivo replication can be autonomous (as for a cell), with the assistance of host factors (as for a virus) or with the assistance of both host and helper virus (as for a phagemid).
30 Cells, spores or viruses are examples of display packages. The replicable genetic package can be eukaryotic or prokaryotic. A display library is formed by introducing nucleic acids encoding exogenous polypeptides to be displayed into the genome of the display package to form a fusion protein with an endogenous protein that is normally expressed from the outer surface of the display package. Expression of the fusion

protein, transport to the outer surface and assembly results in display of exogenous polypeptides from the outer surface of the genetic package.

A further type of display package comprises a polypeptide bound to a nucleic acid encoding the polypeptide. Such an arrangement can be achieved in several ways. US 5,733,731 describe a method in which a plasmid is engineered to expression a fusion protein comprising a DNA binding polypeptide and a polypeptide to be screened. After expression the fusion protein binds to the vector encoding it though the DNA binding polypeptide component. Vectors displaying fusion proteins are screened for binding to a target, and vectors recovered for further rounds of screening or characterization. In another method, polypeptides are screened as components of display package comprising a polypeptide being screened, and mRNA encoding the polypeptide, and a ribosome holding together the mRNA and polypeptide (see Hanes & Pluckthun, *PNAS* 94, 4937-4942 (1997); Hanes et al., *PNAS* 95, 14130-14135 (1998); Hanes et al, *FEBS Let.* 450, 105-110 (1999); US 5,922,545). mRNA of selected complexes is amplified by reverse transcription and PCR and in vitro transcription, and subject to further screening linked to a ribosome and protein translated from the mRNA. In another method, RNA is fused to a polypeptide encoded by the RNA for screening (Roberts & Szostak, *PNAS* 94, 12297-12302 (1997), Nemoto et al., *FEBS Letters* 414, 405-408 (1997). RNA from complexes surviving screening is amplified by reverse transcription PCR and in vitro transcription. In another methods, antibodies are displayed from the outer surface of yeast (see Boder et al., *PNAS* 97, 10701-10705 (2000); Foote et al., id. at 10679-10681).

The genetic packages most frequently used for display libraries are bacteriophage, particularly filamentous phage, and especially phage M13, Fd and F1. Most work has inserted libraries encoding polypeptides to be displayed into either gIII or gVIII of these phage forming a fusion protein. *See, e.g.*, Dower, WO 91/19818; Devlin, W0 91/18989; MacCafferty, WO 92/01047 (gene III); Huse, W0 92/06204; Kang, WO 92/18619 (gene VIII). Such a fusion protein comprises a signal sequence, usually from a secreted protein other than the phage coat protein, a polypeptide to be displayed and either the gene III or gene VIII protein or a fragment thereof. Exogenous coding sequences are often inserted at or near the N-terminus of gene III or gene VIII although other insertion sites are possible. Some filamentous phage vectors have been engineered to produce a second copy of either gene III or gene

VIII. In such vectors, exogenous sequences are inserted into only one of the two copies. Expression of the other copy effectively dilutes the proportion of fusion protein incorporated into phage particles and can be advantageous in reducing selection against polypeptides deleterious to phage growth. In another variation, exogenous polypeptide sequences are cloned into phagemid vectors which encode a phage coat protein and phage packaging sequences but which are not capable of replication. Phagemids are transfected into cells and packaged by infection with helper phage. Use of phagemid system also has the effect of diluting fusion proteins formed from coat protein and displayed polypeptide with wild type copies of coat protein expressed from the helper phage. *See, e.g.,* Garrard, WO 92/09690.

Eukaryotic viruses can be used to display polypeptides in an analogous manner. For example, display of human heregulin fused to gp70 of Moloney murine leukemia virus has been reported by Han, *et al., Proc. Natl. Acad. Sci. USA* **92**:9747-9751 (1995). Spores can also be used as display packages. In this case, polypeptides are displayed from the outer surface of the spore. For example, spores from *B. subtilis* have been reported to be suitable. Sequences of coat proteins of these spores are provided by Donovan, *et al., J. Mol. Biol.* **196**:1-10 (1987). Cells can also be used as display packages. Polypeptides to be displayed are inserted into a gene encoding a cell protein that is expressed on the cells surface. Bacterial cells including *Salmonella typhimurium, Bacillus subtilis, Pseudomonas aeruginosa, Vibrio cholerae, Klebsiella pneumonia, Neisseria gonorrhoeae, Neisseria meningitidis, Bacteroides nodosus, Moraxella bovis,* and especially *Escherichia coli* are preferred. Details of outer surface proteins are discussed by Ladner, *et al.,* US 5,571,698, and Georgiou, *et al., Nature Biotechnology* **15**:29-34 (1997) and references cited therein. For example, the *lamB* protein of *E. coli* is suitable.

B. Displayed Antibodies

Antibody chains can be displayed in single or double chain form. Single chain antibody libraries can comprise the heavy or light chain of an antibody alone or the variable domain thereof. However, more typically, the members of single-chain antibody libraries are formed from a fusion of heavy and light chain variable domains separated by a peptide spacer within a single contiguous protein. *See e.g.,* Ladner, *et al.,* WO 88/06630; McCafferty, *et al.,* WO 92/01047. Double-chain antibodies are formed by noncovalent association of heavy and light chains or

binding fragments thereof. Double chain antibodies can also form by association of two single chain antibodies, each single chain antibody comprising a heavy chain variable domain, a linker and a light chain variable domain. In such antibodies, known as diabodies, the heavy chain of one single-chain antibody binds to the light chain of the other and vice versa, thus forming two identical antigen binding sites (see Hollinger et al., *Proc. Natl. Acad. Sci. USA* 90, 6444-6448 (1993) and Carter & Merchan, *Curr. Op. Biotech.* 8, 449-454 (1997). Thus, phage displaying single chain antibodies can form diabodies by association of two single chain antibodies as a diabody.

The diversity of antibody libraries can arise from obtaining antibody-encoding sequences from a natural source, such as a nonclonal population of immunized or unimmunized B cells. Alternatively, or additionally, diversity can be introduced by artificial mutagenesis of nucleic acids encoding antibody chains before or after introduction into a display vector. Such mutagenesis can occur in the course of PCR or can be induced before or after PCR.

Nucleic acids encoding antibody chains to be displayed optionally flanked by spacers are inserted into the genome of a display package as discussed above by standard recombinant DNA techniques (see generally, Sambrook, *et al.*, Molecular Cloning, A Laboratory Manual, 2d ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989, incorporated by reference herein). The nucleic acids are ultimately expressed as antibody chains (with or without spacer or framework residues). In phage, bacterial and spore vectors, antibody chains are fused to all or part of the an outer surface protein of the replicable package. Libraries often have sizes of about 10^3, 10^4, 10^6, 10^7, 10^8 or more members.

Double-chain antibody display libraries represent a species of the display libraries discussed above. Production of such libraries is described by, *e.g.*, Dower, US 5,427,908; US 5,580,717, Huse WO 92/06204; Huse, in Antibody Engineering, (Freeman 1992), Ch. 5; Kang, WO 92/18619; Winter, WO 92/20791; McCafferty, W0 92/01047; Hoogenboom WO 93/06213; Winter, *et al., Annu. Rev. Immunol.* **12**:433-455 (1994); Hoogenboom, *et al., Immunological Reviews* **130**:41-68 (1992); Soderlind, *et al., Immunological Reviews* **130**:109-124 (1992). For example, in double-chain antibody phage display libraries, one antibody chain is fused to a phage coat protein, as is the case in single chain libraries. The partner antibody chain is complexed with the first antibody chain, but the partner is not directly linked to a

phage coat protein. Either the heavy or light chain can be the chain fused to the coat protein. Whichever chain is not fused to the coat protein is the partner chain. This arrangement is typically achieved by incorporating nucleic acid segments encoding one antibody chain gene into either gIII or gVIII of a phage display vector to form a fusion protein comprising a signal sequence, an antibody chain, and a phage coat protein. Nucleic acid segments encoding the partner antibody chain can be inserted into the same vector as those encoding the first antibody chain. Optionally, heavy and light chains can be inserted into the same display vector linked to the same promoter and transcribed as a polycistronic message. Alternatively, nucleic acids encoding the partner antibody chain can be inserted into a separate vector (which may or may not be a phage vector). In this case, the two vectors are expressed in the same cell (see WO 92/20791). The sequences encoding the partner chain are inserted such that the partner chain is linked to a signal sequence, but is not fused to a phage coat protein. Both antibody chains are expressed and exported to the periplasm of the cell where they assemble and are incorporated into phage particles.

Typically, only the variable region of human light and heavy chains are cloned from a nonhuman transgenic animal. In such instances, the display vector can be designed to express heavy and light chain constant regions or fragments thereof in-frame with heavy and light chain variable regions expressed from inserted sequences. Typically, the constant regions are naturally occurring human constant regions; a few conservative substitutions can be tolerated but are not preferred. In a Fab fragment, the heavy chain constant region usually comprises a C_H1 region, and optionally, part or all of a hinge region, and the light chain constant region is an intact light chain constant region, such as C_κ or C_λ. Choice of constant region isotype depends in part on whether complement-dependent cytotoxity is ultimately required. For example, human isotypes IgG1 and IgG4 support such cytotoxicity whereas IgG2 and IgG3 do not. Alternatively, the display vector can provide nonhuman constant regions. In such situations, typically, only the variable regions of antibody chains are subsequently subcloned from display vectors and human constant regions are provided by an expression vector in frame with inserted antibody sequences.

In a further variation, both constant and variable regions can be cloned from the transgenic animal. For example, heavy chain variable regions can be cloned linked to the C_H1 constant region and light chain variable regions linked to an intact

light chain constant region for expression of Fab fragments. In this situation, display vectors need not encode constant regions.

Antibody encoding sequences can be obtained from lymphatic cells of a nonhuman transgenic animal. Typically, the cells have been immunized, in which case immunization can be performed *in vivo* before harvesting cells, or *in vitro* after harvesting cells, or both. Spleen cells of an immunized animal are a preferred source material. Immunization can be performed with any type of antigen. Antigens are often human proteins.

Rearranged immunoglobulin genes can be cloned from genomic DNA or mRNA. For the latter, mRNA is extracted from the cells and cDNA is prepared using reverse transcriptase and poly dT oligonucleotide primers. Primers for cloning antibody encoding sequences are discussed by Larrick, *et al., Bio/Technology* 7:934 (1989), Danielsson & Borrebaceick, in Antibody Engineering: A Practical Guide (Freeman, NY, 1992), p. 89 and Huse, *id.* at Ch. 5.

Repertoires of antibody fragments have been constructed by combining amplified V_H and V_L sequences together in several ways. Light and heavy chains can be inserted into different vectors and the vectors combined *in vitro* (Hogrefe, *et al., Gene* **128**:119-126 (1993)) or *in vivo* (Waterhouse, *et al., Nucl. Acids. Res.* :2265-66 (1993)). Alternatively, the light and heavy chains can be cloned sequentially into the same vector (Barbas, *et al., Proc. Natl. Acad. Sci. USA* **88**: 7987-82 (1991)) or assembled together by PCR and then inserted into a vector (Clackson, *et al., Nature* **352**:624-28 (1991)). Repertoires of heavy chains can be also be combined with a single light chain or vice versa. Hoogenboom, *et al., J. Mol. Biol.* **227**: 381-88 (1992).

Typically, segments encoding heavy and light antibody chains are subcloned from separate populations of heavy and light chains resulting in random association of a pair of heavy and light chains from the populations in each vector. Thus, modified vectors typically contain combinations of heavy and light chain variable region not found in naturally occurring antibodies. Some of these combinations typically survive the selection process and also exist in the polyclonal libraries described below.

Some exemplary vectors and procedures for cloning populations of heavy chain and light chain encoding sequences have been described by Huse, WO 92/06204. Diverse populations of sequences encoding H_c polypeptides are cloned

into M13IX30 and sequences encoding L_c polypeptides are cloned into M13IX11. The populations are inserted between the *XhoI-SeeI* or *StuI* restriction enzyme sites in M13IX30 and between the *SacI-XbaI* or *EcoRV* sites in M13IX11 (Figures 1A and B of Huse, respectively). Both vectors contain two pairs of *MluI-HindIII* restriction enzyme sites (Figures 1A and B of Huse) for joining together the H_c and L_c encoding sequences and their associated vector sequences. The two pairs are symmetrically orientated about the cloning site so that only the vector proteins containing the sequences to be expressed are exactly combined into a single vector.

Optionally, antibody-encoding sequences can be subjected to artificial mutagenesis before screening to augment the effect of natural somatic mutations. Mutagenesis can be performed by amplifying nucleic acids encoding antibody chains under conditions of mutagenic PCR, or by using mutagenic primers or using uracil templates. Optionally, nucleic acids encoding antibodies can be shuffled with each other, and/or random oligonucleotides as described by Stemmer, U6,117,679.

Others exemplary vectors and procedures for cloning antibody chains into filamentous phage are described in the present Examples.

IV. Enrichment for Polyvalent Display Members

A. Theory of the method

That a display library should preferably be enriched for members displaying multiple copies of a polypeptide is a finding apparently at variance with some early reports in the field. *See, e.g.,* Cwirla *et al., supra.* Most work on display libraries has been done by inserting nucleic acid libraries into pIII or pVIII of filamentous phage. Because pIII is present in 4 or 5 copies per phage and pVIII is present in several hundred copies per phage, some early reports assumed that foreign polypeptides would be displayed in corresponding numbers per phage. However, more recent work has made clear that the actual number of copies of polypeptide displayed per phage is well below theoretical expectations, perhaps due to proteolytic cleavage of polypeptides. Winter, *et al., Ann. Rev. Immunol.* 12:433-55 (1994). Further, vector systems used for phage display often encode two copies of a phage coat protein, one of which is a wild type protein and the other of which forms a fusion protein with exogenous polypeptides to be displayed. Both copies are expressed and the wild type coat protein effectively dilutes the representation of the fusion protein in the phage coat.

A typical ratio of displayed Fabs per phage, when Fabs are expressed from pVIII of a filamentous phage is about 0.2. The probability, Pr(y), of y Fabs being expressed on a phage particle if the average frequency of expression per phage is n is given by the Poisson probability distribution

$$Pr(y)=e^{-n}n^{y}/y!$$

For a frequency of 0.2 Fabs per phage, the probabilities for the expression of 0, 1, 2, and 3 Fabs per phage are 0.82, 0.16, 0.016, and 0.0011. The proportion of phage particle displaying two or more Fabs is therefore only 0.017.

The low representation of members displaying more than one Fab fragment in a phage display library can be related to the result that only a small percentage of such library members are capable of surviving affinity selection to immobilized binding partners. A library was constructed in which all members encoded the same Fab fragment which was known to have a high binding affinity for a particular target. It was found that even under the mildest separation conditions for removal of free from bound phage, it was not possible to bind more than about 0.004 of the total phage. This proportion is the same order of magnitude as the proportion of phage displaying at least two Fab fragments, suggesting that phage must display at least two Fab fragments to bind to immobilized target. Probably shear forces dissociate phage displaying only a single Fab fragment from the solid phase. Therefore, at least two binding events are necessary for a phage-Fab library member to be bound to immobilized target with sufficient avidity to enable separation of the bound from the free phage. It is expected that similar constraints apply in other forms of display library.

Therefore, a preferred strategy of the present invention is to enrich for library members binding to a receptor fused to displayed antibody chains before the library is contacted with a screening target. It is believed that the prescreening enriches for library members displaying at least two copies of a tag and therefore at least two copies of an antibody chain linked to the tag. Library members lacking two or more antibody chains, which are incapable of surviving affinity selection via binding through displayed antibody chain to any immobilized screening target, but which nevertheless can survive affinity selection by formation of multiple nonspecific bonds to such a target or its support, are thus substantially eliminated before screening of the library to the target is performed (see WO98/47343).

B. Tags and Receptors

The above strategy is effected by the use of paired tags and receptors. A tag can any peptide sequence that is common to different members of the library, heterologous to the display package, and fused to a polypeptide displayed from the display package. For example, a tag can be a synthetic peptide sequence, a constant region of an antibody. In some methods, single chain antibodies are displayed in which only the light or heavy chain variable region but not both varies between members. In such situations, among others, the variable region that is the same in different members can be used as a tag. Suitable tag-receptor combinations include epitope and antibody; for example, many high affinity hexapeptide ligands are known for the anti-dynorphin mAb 32.39, (see Barrett *et al., Neuropeptides* **6**:113-120 (1985) and Cull *et al., Proc. Nat'l Acad. Sci. USA* **89**:1865-1869 (1992)) and a variety of short peptides are known to bind the MAb 3E7 (Schatz, *Biotechnology* **11**:1138-43 (1993)). Another combination of tag and antibody is described by Blanar & Rutter, *Science* **256**:1014-1018 (1992).

Another example of a tag-receptor pair is the FLAG™ system (Kodak). The FLAG™ molecular tag consists of an eight amino acid FLAG peptide marker that is linked to the target binding moiety. A 24 base pair segment containing a FLAG coding sequence can be inserted adjacent to a nucleotide sequence that codes for the displayed polypeptide. The FLAG peptide includes an enterokinase recognition site that corresponds to the carboxyl-terminal five amino acids. Capture moieties suitable for use with the FLAG peptide marker include antibodies Anti-FLAG M1, M2 and M5, which are commercially available.

Still other combinations of peptides and antibodies can be identified by conventional phage display methods. Further suitable combinations of peptide sequence and receptor include polyhistidine and metal chelate ligands containing Ni^{2+} immobilized on agarose (see Hochuli in Genetic Engineering: Principles and Methods (ed. JK Setlow, Plenum Press, NY), Ch. 18, pp. 87-96 and maltose binding protein (Maina, *et al., Gene* 74:365-373 (1988)).

Receptors are often labeled with biotin allowing the receptors to be immobilized to an avidin-coated support. Biotin labeling can be performed using the biotinylating enzyme, BirA (*see, e.g.,* Schatz, *Biotechnology* **11**:1138-43 (1993)).

A nucleic acid sequence encoding a tag is inserted into a display vector in such a manner that the tag is expressed as part of the fusion protein containing the

polypeptide to be displayed and an outer surface protein of the display package. The relative ordering of these components is not critical provided that the tag and polypeptide to be displayed are both exposed on the outer surface of the package. For example, the tag can be placed between the outer surface protein and the displayed polypeptide or at or near the exposed end of the fusion protein.

In display packages displaying Fabs, a tag can be fused to either the heavy or the light Fab chain, irrespective which chain is linked to a phage coat protein. Optionally, two different tags can used one fused to each of the heavy and light chains. One tag is usually positioned between the phage coat protein and antibody chain linked thereto, and the other tag is positioned at either the N- or C-terminus of the partner chain.

C. Selection of Polyvalent Library members Members

Selection of polyvalent library members is performed by contacting the library with the receptor for the tag component of library members. Usually, the library is contacted with the receptor immobilized to a solid phase and binding of library members through their tag to the receptor is allowed to reach equilibrium. The complexed receptor and library members are then brought out of solution by addition of a solid phase to which the receptor bears affinity (*e.g.*, an avidin-labeled solid phase can be used to immobilize biotin-labeled receptors). Alternatively, the library can be contacted with receptor in solution and the receptor subsequently immobilized. The concentration of receptor should usually be at or above the Kd of the tag/receptor during solution phase binding so that most displayed tags bind to a receptor at equilibrium. When the receptor-library members are contacted with the solid phase only the library members linked to receptor through at least two displayed tags remain bound to the solid phase following separation of the solid phase from library members in solution. Library members linked to receptor through a single tag are presumably sheared from the solid phase during separation and washing of the solid phase. After removal of unbound library members, bound library members can be dissociated from the receptor and solid phase by a change in ionic strength or pH, or addition of a substance that competes with the tag for binding to the receptor. For example, binding of metal chelate ligands immobilized on agarose and containing Ni^{2+} to a hexahistidine sequence is easily reversed by adding imidazole to the solution to

compete for binding of the metal chelate ligand. Antibody-peptide binding can often be dissociated by raising the pH to 10.5 or higher.

The average number of polypeptides per library member selected by this method is affected by a number of factors. Decreasing the concentration of receptor during solution-phase binding has the effect of increasing the average number of polypeptides in selected library members. An increase in the stringency of the washing conditions also increases the average number of polypeptides per selected library member. The physical relationship between library members and the solid phase can also be manipulated to increase the average number of polypeptides per library member. For example, if discrete particles are used as the solid phase, decreasing the size of the particles increases the steric constraints of binding and should require a higher density of polypeptides displayed per library member.

For Fab libraries having two tags, one linked to each antibody chain, two similar rounds of selection can be performed, with the products of one round becoming the starting materials for the second round. The first round of selection is performed with a receptor to the first tag, and the second round with a receptor to the second tag. Selecting for both tags enriches for library members displaying two copies of both heavy and light antibody chains (*i.e.*, two Fab fragments).

Although the theory underlying the above methods of polyvalent enrichment is believed to be correct, the practice of the invention is in no way dependent on the correctness of this theory. Prescreening a display library for members binding to a tag, followed by screening those members for binding to a target results in a higher degree of enrichment for members with affinity for a target than if the method is performed without the prescreening step. Thus, the method can be practiced as described, and achieve the desired result of highly enriched libraries without any understanding of the underlying mechanism.

D. Selection For Affinity to Target

Library members displaying antibody chains, with or without prescreening to a tag receptor, are screened for binding to a target. The target can be any molecule of interest for which it is desired to identify binding partners. The target should lack specific binding affinity for the tag(s) (if used), because in this step it is the displayed polypeptides being screened, and not the tags that bind to the target. The screening procedure at this step is closely analogous to the prescreening step

except that the affinity reagent is a target of interest rather than a receptor to a tag. The enriched library members are contacted with the target which is usually labeled (*e.g.*, with biotin) in such a manner that allows its immobilization. Binding is allowed to proceed to equilibrium and then target is brought out of solution by contacting with the solid phase in a process known as panning (Parmley & Smith, *Gene* 73:305-318 (1988)). Library members that remain bound to the solid phase throughout the selection process do so by virtue of polyvalent bonds between them and immobilized target molecules. Unbound library members are washed away from the solid phase. In some methods, library members are screened by binding to cells displaying a receptor of interest. Thereafter, the entire cell population can be recovered by centrifugation or fractions bound to phage can be isolated by labelling with a phage specific antibody and separating labelled phage bound to cells using magnetic beads or FACS™. Screening can also be performed for membrane bound proteins using a preparation of phospholipid bearing the antigen. Complexes between the phospholipid and phage can be precipitated using wheat germ agluttin to bind lectins in the phospholipid. Alternatively, phospholipids can be labelled with biotin, and complexes precipitated using avidin-labelled beads.

Usually, library members are subject to amplification before performing a subsequent round of screening. Often, bound library members can be amplified without dissociating them from the support. For example, gene VIII phage library members immobilized to beads, can be amplified by immersing the beads in a culture of *E. coli*. Likewise, bacterial display libraries can be amplified by adding growth media to bound library members. Alternatively, bound library members can be dissociated from the solid phase (*e.g.*, by change of ionic strength or pH) before performing subsequent selection, amplification or propagation.

After affinity selection, bound library members are now enriched for antibody chains having specific affinity for the target of interest (and for polyvalent display members if a prescreening step has been performed). After subsequent amplification, to produce a secondary library, the secondary library remains enriched for display of polypeptides having specific affinity for the target, but, as a result of amplification, is no longer enriched for polyvalent display of polypeptides. Thus, a second cycle of polyvalent enrichment can then be performed, followed by a second cycle of affinity enrichment to the screening target. Further cycles of affinity enrichment to the screening target, optionally, alternating with amplification and

enrichment for polyvalent display can then be performed, until a desired degree of enrichment has been achieved. In some methods, some but not all cycles of affinity screening are preceded by polyvalent enrichment. For example, a first cycle can be performed with affinity enrichment for a target alone, second and third cycles with both polyvalent enrichment and affinity enrichment, and a fourth cycle with just enrichment to the target. Two cycles of polyvalent enrichment are often sufficient.

In a variation, affinity screening to a target is performed in competition with a compound that resembles but is not identical to the target. Such screening preferentially selects for library members that bind to a target epitope not present on the compound. In a further variation, bound library members can be dissociated from the solid phase in competition with a compound having known crossreactivity with a target for an antigen. Library members having the same or similar binding specificity as the known compound relative to the target are preferentially eluted. Library members with affinity for the target through an epitope distinct from that recognized by the compound remain bound to the solid phase.

Discrimination in selecting between antibody chains of different monovalent affinities for the target is affected by the valency of library members and the concentration of target during the solution phase binding. Assuming a minimum of i labeled target molecules must be bound to a library member to immobilize it on a solid phase, then the probability of immobilization can be calculated for a library member displaying n polypeptides. From the law of mass action, the bound/total antibody chain fraction, F, is K[targ]/ (1+K[targ]), where [targ] is the total target concentration in solution. Thus, the probability that i or more displayed antibody chains per library member are bound by the labeled target is given by the binomial probability distribution:

$$\sum_{y=i}^{n} (n!/[y!(n-y)!]\, F^{y}\, (1-F)^{n-y}$$

As the probability is a function of K and [target], multivalent display members each having a monovalent affinity, K, for the target can be selected by varying the concentration of target. The probabilities of solid-phase immobilization for i= 1, 2, or 3, with library members exhibiting monovalent affinities of 0.1/[Ag], 1/[Ag], and 10/[Ag], and displaying n polypeptides per member are:

Probability of Immobilization (i=1)

n	K= 0.1/[targ]	K= 1/[targ]	K= 10/[targ]
1	0.09	0.5	0.91
2	0.17	0.75	0.99
3	0.25	0.875	
4	0.32	0.94	
5	0.38	0.97	
6	0.44	0.98	
7	0.49	0.99	
8	0.53		
9	0.58		
10	0.61		
20	0.85		
50	0.99		

Probability of Immobilization (i=2)

n	K= 0.1/[targ]	K= 1/[targ]	K= 10/[targ]
2	0.008	0.25	0.83
3	0.023	0.50	0.977
4	0.043	0.69	0.997
5	0.069	0.81	
6	0.097	0.89	
7	0.128	0.94	
8	0.160	0.965	
9	0.194	0.98	
20	0.55		
50	0.95		

Probability of Immobilization (i=3)

n	K= 0.1/[targ]	K= 1/[targ]	K= 10/[targ]
3	0.00075	0.125	0.75
4	0.0028	0.31	0.96
5	0.0065	0.50	0.99
6	0.012	0.66	

7	0.02	0.77
8	0.03	0.855
9	0.0415	0.91
10	0.055	0.945
12	0.089	0.98
14	0.128	0.99
20	0.27	
50	0.84	

The above tables show that the discrimination between immobilizing polypeptides of different monovalent binding affinities is affected by the valency of library members (n) and by the concentration of target for the solution binding phase. Discrimination is maximized when n (number of polypeptides displayed per phage) is equal to i (minimum valency required for solid phase binding). Discrimination is also increased by lowering the concentration of target during the solution phase binding. Usually, the target concentration is around the Kd of the polypeptides sought to be isolated. Target concentration of 10^{-8}-10^{-10} M are typical.

Enriched libraries produced by the above methods are characterized by a high proportion of members encoding polypeptides having specific affinity for the target. For example, at least 10, 25, 50, 75, 80, 90, 95, or 99% of members encode antibody chains having specific affinity for the target. In some libraries, at least 10, 25, 50, 75, 80, 90, 95, or 99% of members have affinities of at least 10^8 M^{-1}, 10^9 M^{-1} or 10^{10} M^{-1}, 10^{11} M^{-1}, 10^{12} M^{-1}, 10^{-13} M^{-1}. In some libraries, at least 90, 95 or 99% of nucleic acids encoding antibody heavy chains encode heavy chains of IgG isotype. In some libraries, the nucleic acids encoding heavy chains of members having specific affinity for the target have a median of at least 5, 10, 14, 15, 20 or 25 somatic nucleotide mutations per chain. In some libraries, the nucleic acids encoding light chains of members having specific affinity for the target have a median of a least of 2, 3, 5, 10, 15, 20 or 25 somatic nucleotide mutations per chain. In libraries of double chain antibodies, a pair of segments encoding heavy and light chains of an antibody is considered a library member. The exact percentage of members having affinity for the target depends whether the library has been amplified following selection, because amplification increases the representation of genetic deletions. However, among members with full-length polypeptide coding sequences, the proportion encoding

polypeptides with specific affinity for the target is very high (e.g., at least 50, 75, 80, 90, 95 or 99% having affinity of 10^8 M^{-1}, 10^9 M^{-1} , 10^{10} M^{-1},10^{11} M^{-1}, 10^{12} M^{-12}, 10^{-13} M^{-1} Not all of the library members that encode an antibody chain with specific affinity for the target necessarily display the antibody chain. For example, in a library in which 95% of members with full-length coding sequences encode antibody chains with specific affinity for the target, usually fewer than half actually display the antibody chain. Usually, such libraries have at least 4, 10, 20, 50, 100, 1000, 10,000 or 100,000 different coding sequences. Usually, the representation of any one such coding sequences is no more than 50%, 25% or 10% of the total coding sequences in the library.

E. Variations

1.. Generation of Normalized Display Libraries

A complex antigen, such as a protein molecule, comprises multiple distinct non-overlapping epitopes. However, the individual epitopes of a given antigen are not accessed with equal frequency by the humoral immune system. Instead, particular epitopes can dominate an antibody response. This can result in biased display libraries and biased polyclonal reagents. However, the selection process described above can be manipulated so as to normalize the display library and correct for this natural bias.

Normalized libraries are generated in an iterative process whereby a first non-normalized library is generated, and a non-normalized polyclonal reagent is produced. This non-normalized polyclonal reagent is then mixed with derivatized (e.g. biotinylated) antigen at near stoichiometric concentrations to produce a treated antigen preparation. The non-normalized polyclonal reagent comprises a high concentration of antibody binding species that recognize (and mask) dominant epitopes. Antibody binding species recognizing rare epitopes are found at lower concentrations in the non-normalized polyclonal reagent. For this reason, the treated antigen preparation is depleted of unmasked dominant epitopes relative to an untreated antigen preparation. If this treated antigen preparation is then used to select a new display library; the new library is relatively enriched for antibodies that are not blocked by antibodies that recognize dominant epitopes. Thus the new library, and polyclonal reagents generated from the new library, are normalized. These normalized reagents are useful for characterization of novel antigens in immunohistochemical and functional assays where epitope dominance could

otherwise lead to the generation of reagents that missed particular interesting epitopes. Such rare epitopes in some instances represent neutralizing or agonist epitopes that reveal the biological role of the antigen and the usefulness of antibody reagents for intervening in disease processes mediated by the antigen. Rare epitopes in some instances also reveal otherwise hidden patterns of gene expression due to cell type specific splicing or processing of transcripts or gene products.

The concentration of non-normalized polyclonal reagent used to treat the antigen preparation can be selected using a model antigen. The model antigen can be, for example, an equimolar mixture of four different antigens with different immunogenicities (e.g. keyhole limpet hemocyanin, tetanus toxoid, ovalbumin, and hen eggwhite lysozyme). Mice are immunized with this mixture and an initial non-normalized display library is made from spleen RNA and selected using an equimolar mixture of the same four antigens at a concentration of 1 to 10 nM. Prior to use, the selecting antigens are biotinylated to allow for selection on avidin coated magnetic beads (Binding pairs other than biotin and avidin can be used to derivatize the antigen and magnetic beads respectively. Antibody antigen pairs are suitable, particularly antibodies to small molecule hatpins that can be readily used to derivative the antigen. Antibodies to the antigen itself can also be used, and if these antibodies themselves are biotinylated directly, avidin coated magnetic beads can be used for selection. Because monoclonal antibodies bias the selection against cross-blocking epitopes, it is in some cases desirable to use a polyclonal reagent for this purpose). This initial library is then used to generate a non-normalized polyclonal reagent. The non-normalized polyclonal reagent is then used to treat the biotinylated antigen mixture. Five different preparations of treated antigen are prepared at molar ratios of antigen to polyclonal of 1:10, 1:3, 1:1, 3:1, and 1:10. Each of these five treated antigen preparations is then used to select new display libraries made from the original immunized spleen RNA. Individual clones from the original non-normalized library and the five new normalized libraries are then tested in microtiter, or western dot blot formats for reactivity with each of four individual molecules used in the antigen mixture. The frequency of clonal reactivity to each of the four different antigens will be skewed towards the more immunogenic antigens such as keyhole limpet hemocyanin, and away from less immunogenic antigens such as ovalbumin, in the non-normalized libraries. In optimally normalized libraries the frequencies of clonal reactivity for each of the four antigens will be closer to each other. Thus, the resulting

data can be used to select the optimal ratio of antigen to polyclonal for depleting clones recognizing dominant epitopes, and enriching for biding species recognizing rare epitopes.

2. Selection of Display Libraries for Binding to Cell Surface Antigens

It is sometimes desirable to select display libraries for recognition of cell surface antigens that cannot be easily isolated and purified. Often, purified antigen is either unavailable, or the purification process alters the antigen so as to mask or destroy the desired epitope found on natural cells. It is then useful to be able to select phage libraries directly for recognition of antigen on whole cells. Phage display particles from libraries generated from mice immunized with whole cells can be mixed directly with the same, or different whole cells, and the bound phage separated from unbound phage by precipitation or filtration. Non-specific binding can be reduced by prior clearing with a cell preparation that does not express the desired antigen target or epitope target. For example, mice are immunized with whole cells that have been transfected with a gene encoding an antigen target expressed on the cell surface. The RNA from the immunized mouse is then used to generate a display library, which is first exposed to the parent un-transfected cell line, precleared, then exposed to the transfected cell line, and bound phage particles recovered. Alternatively, the mouse is immunized with whole human cells from tissue affected by a particular disease such as cancer or rheumatoid arthritis. The resulting display library could then be precleared using cells from unaffected tissue to obtain a library enriched for antigens associated with the diseased cells.

The library can also be enriched for desired binding species by simultaneous cross-blocking followed by differential selection. For example, mice are immunized with whole human peripheral blood lymphocytes (PBL), and the immunized spleen RNA used to generate a library. The library is then selected by exposure to whole human PBL that is treated with a biotinylated antibody to a specific desired PBL subset such as the T cell antigen CD4. Phage particles binding to the CD4 positive subset are then selected with avidin coated magnetic beads. Alternatively, other separation techniques, such as flow cytometric sorting, can be used to enrich for binding to specific cell subsets. In this case the antibody to the specific cell subset would either be directly conjugated to a fluorescent dye, or avidin conjugated fluorescent dye would be used as a second step reagent to mark the cells.

Solubilized unpurified antigen preparations can also be used to select display libraries for binding to cell surface antigens. Whole intact cells are first chemically modified to attach biotin (or some other derivative) specifically to proteins accessible on the cell surface. The cells are then disrupted with a mild detergent and/or mild proteolysis to solubilize the bound proteins. The crude solubilized protein preparation can then be used directly to select a display library using avidin (or other binding molecule recognizing the derivatized antigens) coated magnetic beads. The crude preparation can also be further purified prior to the selection step. This method may be advantageous because it provides for selection of the library based on monovalent affinity, as described above.

V. Subcloning Antibody Chains into an Expression Vector

Screening of display library members typically results in a subpopulation of library members having specific affinity for a target. There are a number of options at this point. In some methods, clonal isolates of library members are obtained, and these isolates used directly. In other methods, clonal isolates of library member are obtained, and DNA encoding antibody chains amplified from each isolate. Typically, heavy and light chains are amplified as components of the same DNA molecule before transfer to an expression vector, such that combinations of heavy and light chain existing in the display vector are preserved in the expression vector. For displayed antibody chains that include both human variable regions and human constant regions, typically nucleic acids encoding both the variable region and constant region are subcloned. In other methods, nucleic acids encoding antibody chains are amplified and subcloned en masse from a pool of library members into multiple copies of an expression vector without clonal isolation of individual members.

The subcloning process is now described in detail for transfer of a mixed population of nucleic acids from a display vector to an expression vector. Essentially the same process can be used on nucleic acids obtained from a clonal isolate of an individual display vector.

Nucleic acids encoding antibody chains to be subcloned can be excised by restriction digestion of flanking sequences or can be amplified by PCR using primers to sites flanking the coding sequences. See generally PCR Technology: Principles and Applications for DNA Amplification (ed. H.A. Erlich, Freeman Press,

NY, NY, 1992); PCR Protocols: A Guide to Methods and Applications (eds. Innis, *et al.*, Academic Press, San Diego, CA, 1990); Mattila, *et al., Nucleic Acids Res.* **19**:967 (1991); Eckert, *et al., PCR Methods and Applications* 1:17 (1991); PCR (eds. McPherson et al., IRL Press, Oxford). PCR primers can contain a marker sequence that allows positive selection of amplified fragments when introduced into an expression vector. PCR primers can also contain restriction sites to allow cloning into an expression vector, although this is not necessary. For Fab libraries, if heavy and light chains are inserted adjacent or proximate to each other in a display vector, the two chains can be amplified or excised together. For some Fab libraries, only the variable domains of antibody chain(s) are excised or amplified. If the heavy or light chains of a Fab library are excised or amplified separately, they can subsequently be inserted into the same or different expression vectors.

Having excised or amplified fragments encoding displayed antibody chains, the fragments are usually size-purified on an agarose gel or sucrose gradient. Typically, the fragments run as a single sharp full-length band with a smear at lower molecular corresponding to various deleted forms of coding sequence. The band corresponding to full-length coding sequences is removed from the gel or gradient and these sequences are used in subsequent steps.

The next step is to join the nucleic acids encoding full-length coding sequences to an expression vector thereby creating a population of modified forms of the expression vector bearing different inserts. This can be done by conventional ligation of cleaved expression vector with a mixture of inserts cleaved to have compatible ends. Alternatively, the use of restriction enzymes on insert DNA can be avoided. This method of cloning is beneficial because naturally encoded restriction enzyme sites may be present within insert sequences, thus, causing destruction of the sequence when treated with a restriction enzyme. For cloning without restricting, a mixed population of inserts and linearized vector sequences are treated briefly with a 3' to 5' exonuclease such as T4 DNA polymerase or exonuclease III. See Sambrook, *et al.*, Molecular Cloning, A Laboratory Manual (2nd Ed., CSHP, New York 1989). The protruding 5' termini of the insert generated by digestion are complementary to single-stranded overhangs generated by digestion of the vector. The overhangs are annealed, and the re-annealed vector transfected into recipient host cells. The same result can be accomplished using 5' to 3' exonucleases rather than a 3' to 5' exonuclease.

Preferably, ligation of inserts to expression vector is performed under conditions that allow selection against re-annealed vector and uncut vector. A number of vectors containing conditional lethal genes that allow selection against re-annealed vector under nonpermissive conditions are known. *See, e.g.*, Conley & Saunders, *Mol. Gen. Genet.* **194**:211-218 (1984). These vectors effectively allow positive selection for vectors having received inserts. Selection can also be accomplished by cleaving an expression vector in such a way that a portion of a positive selection marker (*e.g.*, antibiotic resistance) is deleted. The missing portion is then supplied by full-length inserts. The portion can be introduced at the 3' end of polypeptide coding sequences in the display vector, or can be included in a primer used for amplification of the insert. An exemplary selection scheme, in which inserts supply a portion of a tetracycline-resistance gene promoter deleted by *HindIII* cleavage of a pBR-derivative vector, is described in Example 14.

The choice of expression vector depends on the intended host cells in which the vector is to be expressed. Typically, the vector includes a promoter and other regulatory sequences in operable linkage to the inserted coding sequences that ensure the expression of the latter. Use of an inducible promoter is advantageous to prevent expression of inserted sequences except under inducing conditions. Inducible promoters include arabinose, lacZ, metallothionein promoter or a heat shock promoter. Cultures of transformed organisms can be expanded under noninducing conditions without biasing the population for coding sequences whose expression products are better tolerated by the host cells. The vector may also provide a secretion signal sequence position to form a fusion protein with polypeptides encoded by inserted sequences, although often inserted polypeptides are linked to a signal sequences before inclusion in the vector. Vectors to be used to receive sequences encoding antibody light and heavy chain variable domains sometimes encode constant regions or parts thereof that can be expressed as fusion proteins with inserted chains thereby leading to production of intact antibodies or fragments thereof. Typically, such constant regions are human. Conservative mutations although not preferred can be tolerated. For example, if display packages display a heavy chain variable region linked to a C_H1 constant region and a light chain variable region linked to an intact light chain constant region, and the complete antibody chains are transferred from the display vector to the expression vector, then the expression vector can be designed to encode human heavy chain constant region hinge, C_H2 and C_H3 regions in-frame

with the C_H1 region of the inserted heavy chain nucleic acid thereby resulting in expression of an intact antibody. Of course, many minor variations are possible as to precisely which segment of the human heavy chain constant region is supplied by the display package and which by the expression vector. For example, the display package can be designed to include a C_H1 region, and some or all of the hinge region. In this case, the expression vector is designed to supply the residual portion of the hinge region (if any) and the C_H2 and C_H3 regions for expression of intact antibodies.

E. coli is one prokaryotic host useful particularly for cloning the polynucleotides of the present invention. Other microbial hosts suitable for use include bacilli, such as *Bacillus subtilis*, and other enterobacteriaceae, such as *Salmonella*, *Serratia*, and various *Pseudomonas* species. In these prokaryotic hosts, one can also make expression vectors, which typically contain expression control sequences compatible with the host cell (*e.g.*, an origin of replication). In addition, any number of a variety of well-known promoters will be present, such as the lactose promoter system, a tryptophan (trp) promoter system, a beta-lactamase promoter system, or a promoter system from phage lambda. The promoters typically control expression, optionally with an operator sequence, and have ribosome binding site sequences and the like, for initiating and completing transcription and translation.

Other microbes, such as yeast, are also used for expression. Saccharomyces is a preferred host, with suitable vectors having expression control sequences, such as promoters, including 3-phosphoglycerate kinase or other glycolytic enzymes, and an origin of replication, termination sequences and the like as desired. Insect cells in combination with baculovirus vectors can also be used.

Mammalian tissue cell culture can also be used to express and produce the polypeptides of the present invention (see Winnacker, From Genes to Clones (VCH Publishers, N.Y., N.Y., 1987). A number of suitable host cell lines capable of secreting intact immunoglobulins have been developed including the CHO cell lines, various Cos cell lines, HeLa cells, myeloma cell lines, transformed B-cells and hybridomas. Expression vectors for these cells can include expression control sequences, such as an origin of replication, a promoter, and an enhancer (Queen, *et al., Immunol. Rev.* **89**:49-68 (1986)), and necessary processing information sites, such as ribosome binding sites, RNA splice sites, polyadenylation sites, and transcriptional terminator sequences. Preferred expression control sequences are promoters derived

from immunoglobulin genes, SV40, adenovirus, bovine papilloma virus, or cytomegalovirus.

Methods for introducing vectors containing the polynucleotide sequences of interest vary depending on the type of cellular host. For example, calcium chloride transfection is commonly utilized for prokaryotic cells, whereas calcium phosphate treatment or electroporation may be used for other cellular hosts. (See generally Sambrook, *et al., supra*).

Once expressed, collections of antibodies are purified from culture media and host cells. Usually, antibody chains are expressed with signal sequences and are thus released to the culture media. However, if antibody chains are not naturally secreted by host cells, the antibody chains can be released by treatment with mild detergent. Antibody chains can then be purified by conventional methods including ammonium sulfate precipitation, affinity chromatography to immobilized target, column chromatography, gel electrophoresis and the like (see generally Scopes, Protein Purification (Springer-Verlag, N.Y., 1982)).

The above methods result in novel libraries of nucleic acid sequences encoding antibody chains having specific affinity for a chosen target. The libraries of nucleic acids typically have at least 5, 10, 20, 50, 100, 1000, 10^4 or 10^5 different members. Usually, no single member constitutes more than 25 or 50% of the total sequences in the library. Typically, at least 25, 50%, 75, 90, 95, 99 or 99.9% of library members encode antibody chains with specific affinity for the target molecules. In the case of double chain antibody libraries, a pair of nucleic acid segments encoding heavy and light chains respectively is considered a library member. The nucleic acid libraries can exist in free form, as components of any vector or transfected as a component of a vector into host cells. In some libraries, at least 90, 95 or 99% of nucleic acids encoding antibody heavy chains encode heavy chains of IgG isotype. In some libraries, the nucleic acids encoding heavy chains of members having specific affinity for the target have a median of at least 5, 10, 14, 15, 20 or 25 somatic nucleotide mutations per chain. In some libraries, the nucleic acids encoding light chains of members having specific affinity for the target have a median of a least 2, 3, 5, 10, 15, 20 or 25 somatic nucleotide mutations per chain.

The nucleic acid libraries can be expressed to generate polyclonal libraries of antibodies having specific affinity for a target. The composition of such libraries is determined from the composition of the nucleotide libraries. Thus, such

libraries typically have at least 5, 10, 20, 50, 100, 1000, 10^4 or 10^5 members with different amino acid composition. Usually, no single member constitutes more than 25 or 50% of the total polypeptides in the library. The percentage of antibody chains in an antibody chain library having specific affinity for a target is typically lower than the percentage of corresponding nucleic acids encoding the antibody chains. The difference is due to the fact that not all polypeptides fold into a structure appropriate for binding despite having the appropriate primary amino acid sequence to support appropriate folding. In some libraries, at least 25, 50, 75, 90, 95, 99 or 99.9% of antibody chains have specific affinity for the target molecules. Again, in libraries of multi-chain antibodies, each antibody (such as a Fab or intact antibody) is considered a library member. In some libraries, at least 90, 95 or 99% of heavy chains are of IgG isotype. In some libraries, the heavy chains having specific affinity for the target have a median of at least 1, 2, 3, 4, 5, 7,10, 12, 15, or 20 somatic amino acid mutations per chain. In some libraries, the light chains having specific affinity for the target have a median of a least of 1, 2, 3, 5, 10, 12, 15, 20 somatic amino acid mutations per chain. The different antibody chains differ from each other in terms of fine binding specificity and affinity for the target. Some such libraries comprise members binding to different epitopes on the same antigen. Some such libraries comprises at least two members that bind to the same antigen without competing with each other.

Polyclonal libraries of human antibodies resulting from the above methods are distinguished from natural populations of human antibodies both by the high percentages of high affinity binders in the present libraries, and in that the present libraries typically do not show the same diversity of antibodies present in natural populations. The reduced diversity in the present libraries is due to the nonhuman transgenic animals that provide the source materials not including all human immunoglobulin genes. For example, some polyclonal antibody libraries are free of antibodies having lambda light chains. Some polyclonal antibody libraries of the invention have antibody heavy chains encoded by fewer than 10, 20, 30 or 40 V_H genes. Some polyclonal antibody libraries of the invention have antibody light chains encoded by fewer than 10, 20, 30 or 40 V_L genes.

VI. Diagnostic and Therapeutics Uses

Human antibodies produced by the invention have a number of treatment (both therapeutic and prophylactic), diagnostic and research uses. For example, human antibodies to pathogenic microorganisms can be used for treatment of infections by the organisms. Such antibodies can also be used for diagnosis, either in vivo or in vitro. Antibodies directed against cellular receptors can be used to agonize or antagonize receptor function. For example, antibodies directed against adhesion molecules can be used to reduced undesired immune response. Such antibodies can also be used for in vivo imaging of inflammation. Other antibodies are directed against tumor antigens, and can be used either directly or in combination with an effector molecule for elimination of tumor cells. Antibodies can also be used for diagnosis, either in vitro or in vivo.

Use of polyclonal human antibodies of the invention in diagnostics and therapeutics is particularly advantageous. Use of polyclonals hitherto has been limited by the inability to generate preparations that have a well-defined affinity and specificity. Monoclonal antibodies developed using hybridoma technology do have well-defined specificity and affinity, but the selection process is often long and tedious. Further, a single monoclonal antibody often does not meet all of the desired specificity requirements. Formation of polyclonal mixtures by isolation, and characterization of individual monoclonal antibodies, which are then mixed would be time consuming process which would increase in proportion to the number of monoclonals included in the mixture and become prohibitive for substantial numbers of monoclonal antibodies. The polyclonal libraries of antibodies and other polypeptides having specificity for a given target produced by the present methods avoid these difficulties, and provide reagents that are useful in many therapeutic and diagnostic applications.

The use of polyclonal mixtures has a number of advantages with respect to compositions made of one monoclonal antibody. By binding to multiple sites on a target, polyclonal antibodies or other polypeptides can generate a stronger signal (for diagnostics) or greater blocking/inhibition/cytotoxicity (for therapeutics) than a monoclonal that binds to a single site. Further, a polyclonal preparation can bind to numerous variants of a prototypical target sequence (*e.g.*, allelic variants, species variants, strain variants, drug-induced escape variants) whereas a monoclonal antibody may bind only to the prototypical sequence or a narrower range of variants thereto.

Polyclonal mixture are also particularly useful as reagents for analyzing the function of individual gene products. A single protein can comprise multiple epitopes; and binding of antibody molecules to these different epitopes can have different effects on the ability of the protein to function. For example, a cytokine molecule can have antigenic epitopes within or near the normal receptor binding site. Antibodies that recognize these epitopes may therefore be considered neutralizing because they block receptor binding. These antibodies may therefore be particularly useful for elucidating the normal function of this cytokine. The antibodies can be used in in vivo or in vitro assays to discover the consequences of loss of function for this particular cytokine. However, the same cytokine may comprise additional epitopes that are distant from the normal receptor binding site. Antibodies that bind to these epitopes may fail to neutralize the cytokine. These individual antibodies may then be less useful for determining the normal function of this particular cytokine. It is therefore desirable to perform such assays using polyclonal mixtures of different antibodies to the target molecule. Such mixtures are preferred over monoclonal antibody reagents because they have a higher probability of including neutralizing antibodies. Thus, polyclonal reagents have a higher probability of being informative in assays for determining the normal function of an individual gene product.

Cytokines are not the only class of molecules for which polyclonal reagents are useful for determining normal function. Many different biological molecules are involved in receptor-ligand type binding interactions. Many of these also comprise multiple epitopes, only a fraction of which are within or adjacent to the sites of intermolecular interaction. Polyclonal reagents have a higher probability of blocking these intermolecular interactions than monoclonal reagents. Enzymes will also show different degrees of perturbation from their normal function on binding to different antibodies with different epitope specificities. Thus polyclonal mixtures of antibodies, comprising individual molecules with different epitope specificities, are useful for determining the normal function of biomolecules that comprise multiple epitopes.

Polyclonal mixtures are also important for determining the tissue distribution of individual proteins. Differential RNA splicing, glycosylation and post-translational modifications can mask or eliminate individual epitopes in particular tissues or cell types. Polyclonal mixtures will thus have a higher probability of

including antibodies that recognize target molecules in a broad variety of tissues and cell types than monoclonal reagents which recognize only a single epitope.

In addition, polyclonal reagents are useful for determining the correlation between particular genetic backgrounds, pathologies, or disease states, and the expression of a particular gene product. In this case, the polyclonal reagent can be used to detect the presence of the gene product in samples from a variety of different individuals, each of which could express allelic variants of the gene product that might eliminate particular epitopes.

After a polyclonal reagent has been used either to determine the function of a given target, or to associate the expression of that particular target with a particular pathology. A monoclonal reagent that also recognizes the target can be generated. Particular epitopes are sometimes desired. Epitopes resulting in broad recognition across a population, or epitopes resulting in neutralizing or blocking antibodies, or epitopes resulting in agonist or antagonist antibody molecules. If the desired characteristic was detected in the polyclonal reagent, it may be possible to identify monoclonal antibodies from with the polyclonal pool. This is a particular advantage of using expression libraries to generate the polyclonal reagent. It is relatively simple to isolate and test individual expression clones from the library used to generate the polyclonal reagent. These clones can then be tested individually, or in smaller pools, to find monoclonal antibodies having the desired characteristics. Such monoclonal Fabs can then be expressed in mammalian expression vectors as intact whole human IgG, IgA, IgM, IgD, or IgE antibodies. These whole antibodies may be useful as therapeutic reagents for the treatment of pathologies associated with the target molecule. It is thus desirable to use human immunoglobulin transgenic mice for the construction of the original phage display library. Monoclonal antibodies derived from such animals can be expressed as completely human molecules, and will exhibit reduced immunogenicity.

Individual antibodies or polyclonal preparations of antibodies can be incorporated into compositions for diagnostic or therapeutic use. The preferred form depends on the intended mode of administration and diagnostic or therapeutic application. The compositions can also include, depending on the formulation desired, pharmaceutically-acceptable, non-toxic carriers or diluents, which are defined as vehicles commonly used to formulate pharmaceutical compositions for animal or human administration. The diluent is selected so as not to affect the biological

activity of the combination. Examples of such diluents are distilled water, physiological phosphate-buffered saline, Ringer's solutions, dextrose solution, and Hank's solution. In addition, the pharmaceutical composition or formulation may also include other carriers, adjuvants, or nontoxic, nontherapeutic, nonimmunogenic stabilizers and the like. See Remington's Pharmaceutical Science, (15th ed., Mack Publishing Company, Easton, Pennsylvania, 1980). Compositions intended for in vivo use are usually sterile. Compositions for parental administration are sterile, substantially isotonic and made under GMP condition.

It is apparent from the foregoing that the invention provides for a variety of uses. The invention provides the use of a display method to screen nucleic acids encoding antibody chains obtained from an immunized nonhuman transgenic animal whose genome comprises a plurality of human immunoglobulin genes to produce a highly enriched polyclonal population of human antibodies with high affinity for the immunogen. The above use does not require screening phage displaying antibodies with a random peptide library to select random peptide sequences, and the random peptides are in turn being used to immunize an animal such that further antibodies are generated. The invention further provides for the use of a nucleic acid to immunize a nonhuman transgenic animal whose genome comprises a plurality of human immunoglobulin genes that can be expressed to produce a human antibody to the protein encoded by the nucleic acid. The invention further provides for use of an immunized animal that lacks a detectable titer to the immunogen for the production of antibodies to the immunogen. The invention further provides for the use of enrichment of a population of B cells for a subpopulation expressing antibodies of IgG isotype for the production of a display library containing random combinations of heavy and light chains.

Although the invention has been described in detail for purposes of clarity of understanding, it will be obvious that certain modifications may be practiced within the scope of the appended claims. All publications and patent documents cited in this application are hereby incorporated by reference in their entirety for all purposes to the same extent as if each were so individually denoted. Cell lines producing antibodies CD.TXA.1.PC (ATCC 98388, April 3, 1997), CD.43.9 (ATCC 98390, April 3, 1997), CD.43.5.PC (ATCC 98389, April 3, 1997) and 7F11 (HB-12443, December 5, 1997) have been deposited at the American Type Culture Collection, Rockville, Maryland under the Budapest Treaty on the dates indicated and

given the accession numbers indicated. The deposits will be maintained at an authorized depository and replaced in the event of mutation, nonviability or destruction for a period of at least five years after the most recent request for release of a sample was received by the depository, for a period of at least thirty years after the date of the deposit, or during the enforceable life of the related patent, whichever period is longest. All restrictions on the availability to the public of these cell lines will be irrevocably removed upon the issuance of a patent from the application.

Example 1: Purification of RNA from mouse spleens

Mice having 3 different sets of human heavy chain genes were used to make the antibody phage libraries to interleukin 8. Production of mice is described in Examples 23 and 24. The mice were immunized with interleukin 8 (Example I). Mice were immunized with 25 microgram of antigen at 0.713mg/ml. In a first procedure, mice were immunized once a month beginning with CFA followed by IFA until a high human gamma titer was reached (ca 6500) after a further six weeks, mice were boosted ip on days –7, -6, -5, and sacrificed 5 days later. In an alternative procedure, mice were immunized every two weeks beginning with CFA and followed by IFA. After a high human gamma titer was reached, mice were boosted on days –3, and –2 and sacrificed two days later.

The spleens were harvested in a laminar flow hood and transferred to a petri dish, trimming off and discarding fat and connective tissue. The spleen was, working quickly, macerated with the plunger from a sterile 5 cc syringe in the presence of 1.0 ml of solution D (25.0 g guanidine thiocyanate (Roche Molecular Biochemicals, Indianapolis, IN), 29.3 ml sterile water, 1.76 ml 0.75 M sodium citrate (pH 7.0), 2.64 ml 10% sarkosyl (Fisher Scientific, Pittsburgh, PA), 0.36 ml 2-mercaptoethanol (Fisher Scientific, Pittsburgh, PA)). The spleen suspension was pulled through an 18 gauge needle until viscous and all cells were lysed, then transferred to a microcentrifuge tube. The petri dish was washed with 100 µl of solution D to recover any remaining spleen, and this was transferred to the tube. The suspension was then pulled through a 22 gauge needle an additional 5-10 times. The sample was divided evenly between two microcentrifuge tubes and the following added in order, with mixing by inversion after each addition: 100 µl 2 M sodium acetate (pH 4.0), 1.0 ml water-saturated phenol (Fisher Scientific, Pittsburgh, PA),

200µl chloroform/isoamyl alcohol 49:1 (Fisher Scientific, Pittsburgh, PA). The solution was vortexed for 10 seconds and incubated on ice for 15 min. Following centrifugation at 14 krpm for 20 min at 2-8 °C, the aqueous phase was transferred to a fresh tube. An equal volume of water saturated phenol/chloroform/isoamyl alcohol (50:49:1) was added, and the tube was vortexed for ten seconds. After a 15 min incubation on ice, the sample was centrifuged for 20 min at 2-8 °C, and the aqueous phase was transferred to a fresh tube and precipitated with an equal volume of isopropanol at -20 °C for a minimum of 30 min. Following centrifugation at 14,000 rpm for 20 min at 4 °C, the supernatant was aspirated away, the tubes briefly spun and all traces of liquid removed. The RNA pellets were each dissolved in 300 µl of solution D, combined, and precipitated with an equal volume of isopropanol at -20 °C for a minimum of 30 min. The sample was centrifuged 14, 000 rpm for 20 min at 4 °C, the supernatant aspirated as before, and the sample rinsed with 100 µl of ice-cold 70% ethanol. The sample was again centrifuged 14,000 rpm for 20 min at 4 °C, the 70% ethanol solution aspirated, and the RNA pellet dried in vacuo. The pellet was resuspended in 100µl of sterile distilled water. The concentration was determined by A_{260} using an absorbance of 1.0 for a concentration of 40µg/ml. The RNA was stored at -80 °C.

Example 2: Preparation of complementary DNA (cDNA)

The total RNA purified as described above was used directly as template for cDNA. RNA (50 µg) was diluted to 100 µL with sterile water, and 10 µL-130 ng/µL oligo dT12 (synthesized on Applied Biosystems Model 392 DNA synthesizer at Biosite Diagnostics) was added. The sample was heated for 10 min at 70 °C, then cooled on ice. 40 µL 5 X first strand buffer was added (Gibco/BRL, Gaithersburg, MD), 20 µL 0.1 M dithiothreitol (Gibco/BRL, Gaithersburg, MD), 10 µL 20 mM deoxynucleoside triphosphates (dNTP's, Roche Molecular Biochemicals, Indianapolis, IN), and 10 µL water on ice. The sample was then incubated at 37 °C for 2 min. 10 µL reverse transcriptase (Superscript™ II, Gibco/BRL, Gaithersburg, MD) was added and incubation was continued at 37 °C for 1 hr. The cDNA products were used directly for polymerase chain reaction (PCR).

Example 3: Amplification of human antibody sequence cDNA by PCR

The cDNA of four mice having the genotype HCo7 was amplified using 3-5' oligonucleotides and 1-3' oligonucleotide for heavy chain sequences (Table A), and 10-5' oligonucleotides and 1-3' oligonucleotide for the kappa chain sequences (Table B). The cDNA of one mouse having the genotype HCo12 was amplified using 5-5' oligonucleotides and 1-3' oligonucleotide for heavy chain sequences (Table C), and the oligonucleotides shown in Table B for the kappa chain sequences. The cDNA of two mice having the genotype HCo7/Co12 was amplified using the oligonucleotide sequences shown in Tables A and C for the heavy chain sequences and oligonucleotides shown in Table B for the kappa chain sequences. The 5' primers were made so that a 20 nucleotide sequence complementary to the M13 uracil template was synthesized on the 5' side of each primer. This sequence is different between the H and L chain primers, corresponding to 20 nucleotides on the 3' side of the pelB signal sequence for L chain primers and the alkaline phosphatase signal sequence for H chain primers. The constant region nucleotide sequences required only one 3' primer each to the H chains and the kappa L chains (Tables A and B). Amplification by PCR was performed separately for each pair of 5' and 3' primers. A 50 µL reaction was performed for each primer pair with 50 pmol of 5' primer, 50 pmol of 3' primer, 0.25 µL Taq DNA Polymerase (5 units/µL, Roche Molecular Biochemicals, Indianapolis, IN), 3 µL cDNA (described in Example 2), 5 µL 2 mM dNTP's, 5 µL 10 x Taq DNA polymerase buffer with $MgCl_2$ (Roche Molecular Biochemicals, Indianapolis, IN), and H_2O to 50 µL. Amplification was done using a GeneAmp® 9600 thermal cycler (Perkin Elmer, Foster City, CA) with the following program: 94 °C for 1 min; 30 cycles of 94 °C for 20 sec, 55 °C for 30 sec, and 72 °C for 30 sec; 72 °C for 6 min; 4 °C.

Table A. Heavy chain oligonucleotides used to amplify cDNA for Hco7 mice. Oligonucleotides 188, 944 and 948 are 5' primers and oligonucleotide 952 is the 3' primer.

OLIGO #	5' TO 3' SEQUENCE
188	TT ACC CCT GTG GCA AAA GCC GAA GTG CAG CTG GTG GAG TCT GG

944	TT ACC CCT GTG GCA AAA GCC CAG GTG CAG CTG GTG CAG TCT GG
948	TT ACC CCT GTG GCA AAA GCC CAG GTG CAG CTG GTG GAG TCT GG
952	GA TGG GCC CTT GGT GGA GGC

Table B. Kappa chain oligonucleotides used to amplify cDNA from Hco7 mice, Hco12 mice, and Hco7/Co12 mice. Oligonucleotide 973 is the 3' primer and the rest are 5' primers.

OLIGO #	5' TO 3' SEQUENCE
189	CT GCC CAA CCA GCC ATG GCC GAA ATT GTG CTC ACC CAG TCT CC
931	TC GCT GCC CAA CCA GCC ATG GCC GTC ATC TGG ATG ACC CAG TCT CC
932	TC GCT GCC CAA CCA GCC ATG GCC AAC ATC CAG ATG ACC CAG TCT CC
933	TC GCT GCC CAA CCA GCC ATG GCC GCC ATC CGG ATG ACC CAG TCT CC
934	TC GCT GCC CAA CCA GCC ATG GCC GCC ATC CAG TTG ACC CAG TCT CC
935	TC GCT GCC CAA CCA GCC ATG GCC GAA ATA GTG ATG ACG CAG TCT CC
936	TC GCT GCC CAA CCA GCC ATG GCC GAT GTT GTG ATG ACA CAG TCT CC
937	TC GCT GCC CAA CCA GCC ATG GCC GAA ATT GTG TTG ACG CAG TCT CC
955	TC GCT GCC CAA CCA GCC ATG GCC GAC ATC CAG ATG ATC CAG TCT CC
956	TC GCT GCC CAA CCA GCC ATG GCC GAT ATT GTG ATG ACC CAG ACT CC
973	CAG CAG GCA CAC AAC AGA GGC

Table C. Heavy chain oligonucleotides used to amplify cDNA for Hco12 mice. Oligonucleotides 944, 945, 946, 947 and 948 are 5' primers and oligonucleotide 952 is the 3' primer. The sequences of 944, 948 and 952 are shown in Table A.

OLIGO #	5' TO 3' SEQUENCE
945	TT ACC CCT GTG GCA AAA GCC GAG GTG CAG CTG TTG GAG TCT GG
946	TT ACC CCT GTG GCA AAA GCC GAG GTG CAG CTG GTG CAG TCT GG
947	TT ACC CCT GTG GCA AAA GCC CAG GTG CAG CTA CAG CAG TGG GG

The dsDNA products of the PCR process were then subjected to asymmetric PCR using only 3' primer to generate substantially only the anti-sense strand of the target genes. Oligonucleotide 953 was used as the 3' primer for kappa chain asymmetric PCR (Table D) and oligonucleotide 952 was used as the 3' primer for heavy chain asymmetric PCR (Table A). For each spleen, two asymmetric reactions were run for the kappa chain PCR products to primer 189, 931, 932, 933, 934, 936, 955, and 956, four asymmetric reactions were run for the kappa chain PCR product to primer 935, and eight asymmetric reactions were run for the kappa chain PCR product to primer 937. The number of asymmetric reactions used for each heavy chain PCR product was dependent on the mouse genotype. For Co7 mice, eight asymmetric reactions were run for each PCR product. For Co12 mice, eight asymmetric reactions were run for the PCR product from primer 944, and four asymmetric reactions were run for the PCR products from the other primers. For Co7/Co12 mice, six asymmetric reactions were run for the PCR products from

primers 944 and 948, and three asymmetric reactions were run for the PCR products from the other primers. Each reaction described above is 100 µL total volume with 200 pmol of 3' primer, 2 µL of ds-DNA product, 0.5 µL Taq DNA Polymerase, 10 µL 2 mM dNTP's, 10 µL 10 X Taq DNA polymerase buffer with $MgCl_2$, and H_2O to 100µL. Heavy chain reactions were amplified using the thermal profile described above, while kappa chain reactions were amplified with the same thermal profile but 25 cycles were used instead of 30 cycles.

Table D. Oligonucleotide sequences used for asymmetric PCR of kappa chains.

OLIGO #	5' TO 3' SEQUENCE
953	GAC AGA TGG TGC AGC CAC AGT

Example 4: Purification of ss-DNA by high performance liquid chromatography and kinasing ss-DNA

The H chain ss-PCR products and the L chain ss-PCR products were separately pooled and ethanol precipitated by adding 2.5 volumes ethanol and 0.2 volumes 7.5 M ammonium acetate and incubating at -20 °C for at least 30 min. The DNA was pelleted by centrifuging at 15,000 rpm for 15 min at 2-8 °C. The supernatant was carefully aspirated, and the tubes were briefly spun a 2nd time. The last drop of supernatant was removed with a pipet. The DNA was dried *in vacuo* for 10 min on medium heat. The H chain products were dissolved in 210 µL water and the L chain products were dissolved separately in 210 µL water. The ss-DNA was purified by high performance liquid chromatography (HPLC) using a Hewlett Packard 1090 HPLC and a Gen-Pak™ FAX anion exchange column (Millipore Corp., Milford, MA). The gradient used to purify the ss-DNA is shown in Table 1, and the oven temperature was at 60 °C. Absorbance was monitored at 260 nm. The ss-DNA eluted from the HPLC was collected in 0.5 min fractions. Fractions containing ss-DNA were pooled, ethanol precipitated, pelleted and dried as described above. The dried DNA pellets were resuspended in 200 µL sterile water.

Table 1: HPLC gradient for purification of ss-DNA

Time (min)	%A	%B	%C	Flow (mL/min)
0	70	30	0	0.75
2	40	60	0	0.75
17	15	85	0	0.75

18	0	100	0	0.75
23	0	100	0	0.75
24	0	0	100	0.75
28	0	0	100	0.75
29	0	100	0	0.75
34	0	100	0	0.75
35	70	30	0	0.75

Buffer A is 25 mM Tris, 1 mM EDTA, pH 8.0

Buffer B is 25 mM Tris, 1 mM EDTA, 1 M NaCl, pH 8.0

Buffer C is 40 mm phosphoric acid

The ss-DNA was kinased on the 5' end in preparation for mutagenesis (Example 7). 24 µL 10 x kinase buffer (United States Biochemical, Cleveland, OH), 10.4 µL 10 mM adenosine-5'-triphosphate (Boehringer Mannheim, Indianapolis, IN), and 2 µL polynucleotide kinase (30 units/µL, United States Biochemical, Cleveland, OH) was added to each sample, and the tubes were incubated at 37 °C for 1 hr. The reactions were stopped by incubating the tubes at 70 °C for 10 min. The DNA was purified with one extraction of equilibrated phenol (pH>8.0, United States Biochemical, Cleveland, OH)-chloroform-isoamyl alcohol (50:49:1) and one extraction with chloroform:isoamyl alcohol (49:1). After the extractions, the DNA was ethanol precipitated and pelleted as described above. The DNA pellets were dried, then dissolved in 50 µL sterile water. The concentration was determined by measuring the absorbance of an aliquot of the DNA at 260 nm using 33 µg/mL for an absorbance of 1.0. Samples were stored at -20 °C.

Example 5: Construction of Antibody Phage Display Vector having human antibody constant region sequences.

The antibody phage display vector for cloning antibodies was derived from an M13 vector supplied by Ixsys, designated 668-4. The vector 668-4 contained the DNA sequences encoding the heavy and light chains of a mouse monoclonal Fab fragment inserted into a vector described by Huse, WO 92/06024. The vector had a Lac promoter, a pelB signal sequence fused to the 5' side of the L chain variable region of the mouse antibody, the entire kappa chain of the mouse antibody, an alkaline phosphatase signal sequence at the 5' end of the H chain variable region of the mouse antibody, the entire variable region and the first constant region of the H

chain, and 5 codons of the hinge region of an IgG1 H chain. A decapeptide sequence was at the 3' end of the H chain hinge region and an amber stop codon separated the decapeptide sequence from the pseudo-gene VIII sequence. The amber stop allowed expression of H chain fusion proteins with the gene VIII protein in E. coli suppressor strains such as XL1 blue (Stratagene, San Diego, CA), but not in nonsuppressor cell strains such as MK30 (Boehringer Mannheim, Indianapolis, IN) (see Fig. 1).

To make the first derivative cloning vector, deletions were made in the variable regions of the H chain and the L chain by oligonucleotide directed mutagenesis of a uracil template (Kunkel, *Proc. Natl. Acad. Sci. USA* **82**:488 (1985); Kunkel, *et al., Methods. Enzymol.* **154**:367 (1987)). These mutations deleted the region of each chain from the 5' end of CDR1 to the 3' end of CDR3, and the mutations added a DNA sequence where protein translation would stop (see Fig. 2 for mutagenesis oligonucleotides). This prevented the expression of H or L chain constant regions in clones without an insert, thereby allowing plaques to be screened for the presence of insert. The resulting cloning vector was called BS11.

Many changes were made to BS11 to generate the cloning vector used in the present screening methods. The amber stop codon between the heavy chain and the pseudo gene VIII sequence was removed so that every heavy chain was expressed as a fusion protein with the gene VIII protein. This increased the copy number of the antibodies on the phage relative to BS11. A *HindIII* restriction enzyme site in the sequence between the 3' end of the L chain and the 5' end of the alkaline phosphatase signal sequence was deleted so antibodies could be subcloned into a pBR322 derivative (Example 14). The interchain cysteine residues at the carboxyl-terminus of the L and H chains were changed to serine residues. This increased the level of expression of the antibodies and the copy number of the antibodies on the phage without affecting antibody stability. Nonessential DNA sequences on the 5' side of the lac promoter and on the 3'side of the pseudo gene VIII sequence were deleted to reduce the size of the M13 vector and the potential for rearrangement. A transcriptional stop DNA sequence was added to the vector at the L chain cloning site to replace the translational stop so that phage with only heavy chain proteins on their surface, which might be nonspecifically in panning, could not be made. Finally, DNA sequences for protein tags were added to different vectors to allow enrichment for polyvalent phage by metal chelate chromatography (polyhistidine sequence) or by affinity purification using a decapeptide tag and a magnetic latex having an

immobilized antibody that binds the decapeptide tag. BS45 had a polyhistidine sequence between the end of the heavy chain constant region and the pseudo-gene VIII sequence, and a decapeptide sequence at the 3' end of the kappa chain constant region.

The mouse heavy and kappa constant region sequences were deleted from BS45 by oligonucleotide directed mutagenesis. Oligonucleotide 864 was used to delete the mouse kappa chain and oligonucleotide 862 was used to delete the mouse heavy chain.

Oligonucleotide 864

5' ATC TGG CAC ATC ATA TGG ATA AGT TTC GTG TAC AAA ATG CCA GAC CTA GAG GAA TTT TAT TTC CAG CTT GGT CCC

Oligonucleotide 862

5' GTG ATG GTG ATG GTG ATG GAT CGG AGT ACC AGG TTA TCG AGC CCT CGA TAT TGA GGA GAC GGT GAC TGA

Deletion of both constant region sequences was determined by amplifying the DNA sequence containing both constant regions by PCR using oligonucleotides 5 and 197, followed by sizing the PCR products on DNA agarose gel. The PCR was accomplished as described in Example 3 for the double-stranded DNA, except 1μL of phage was template instead of cDNA. Phage with the desired deletion had a shorter PCR product than one deletion or no deletion. Uracil template was made from one phage stock having both deletions, as described in Example 6. This template, BS46, was used to insert the human constant region sequences for the kappa chain and IgG1.

Primer 5
5' GCA ACT GTT GGG AAG GG

Primer 197
5' TC GCT GCC CAA CCA GCC ATG

The human constant region DNA sequences were amplified from human spleen cDNA (Clontech, Palo Alto, California). Oligonucleotides 869 and 870 were used to amplify the kappa constant region sequence, and oligonucleotides 867 and 876 were used to amplify the IgG1 constant region sequence and the codons for 6

amino acids of the hinge region (Kabat et al., Sequences of Proteins of Immunological Interest, 1991).

5' PCR primer (869)- GGG ACC AAG CTG GAA ATA AAA CGG GCT GTG GCT GCA CCA TCT GTC T

3' PCR primer (870)- ATC TGG CAC ATC ATA TGG ATA AGA CTC TCC CCT GTT GAA GCT CTT

5' PCR primer (867)- TCA GTC ACC GTC TCC TCA GCC TCC ACC AAG GGC CCA TC

3' PCR primer (876)- GTG ATG GTG ATG GTG ATG AGA TTT GGG CTC TGC TTT CTT GTC C

PCR (1-50μL reaction for each chain) was performed using Expand high-fidelity PCR system (Roche Molecular Biochemicals, Indianapolis, IN). Each 50μL reaction contained 50 pmol of 5' primer, 50 pmol of 3' primer, 0.35 units of Expand DNA polymerase, 5μL 2mM dNTP's, 5μL 10 x Expand reaction buffer, 1μL cDNA as template, and water to 50μL. The reaction was carried out in a Perkin-Elmer thermal cycler (Model 9600) using the following thermal profile for the kappa chain: one cycle of denaturation at 94 °C (1 min); ten cycles of denaturation (15 sec, 94 °C), annealing (30 sec, 55 °C), elongation (60 sec, 72 °C); fifteen cycles of denaturation (15 sec, 94 °C), annealing (30 sec, 55 °C), elongation (80 sec plus 20 sec for each additional cycle, 72 °C); elongation (6 min, 72 °C); soak (4 °C, indefinitely). The thermal profile used for the heavy chain reaction had twenty cycles instead of fifteen in the second part of the thermal profile.

The dsDNA products of the PCR process were then subjected to asymmetric PCR using only 3' primer to generate substantially only the anti-sense strand of the human constant region genes, as described in Example 3. Five reactions were done for the kappa chain and ten reactions were done for the heavy chain (100μL per reaction). The thermal profile for both constant region genes is the same as that described in Example 3, including the heavy chain asymmetric PCR was done with 30 cycles and the kappa chain asymmetric PCR was done with 25 cycles. The single stranded DNA was purified by HPLC as described in Example 4. The HPLC purified kappa chain DNA was dissolved in 55μL of water and the HPLC purified heavy chain was dissolved in 100μL of water. The DNA was quantified by absorbance at 260nm, as described in Example 4, then the DNA was kinased as described in Example 4 except added 6μL 10 x kinase buffer, 2.6μL 10 mM ATP, and

0.5μL of polynucleotide kinase to 50μL of kappa chain DNA. Twice those volumes of kinase reagents were added to 100μL of heavy chain DNA.

The kinased DNA was used to mutate BS46 without purifying the DNA by extractions. The mutagenesis was performed on a 2 μg scale by mixing the following in a 0.2 mL PCR reaction tube: 8 μl of (250 ng/μl) BS46 uracil template, 8 μl of 10 x annealing buffer (200 mM Tris pH 7.0, 20 mM $MgCl_2$, 500 mM NaCl), 2.85 μl of kinased single-stranded heavy chain insert (94 ng/μl) ,6.6 μl of kinased single-stranded kappa chain insert (43.5 ng/μl), and sterile water to 80 μl. DNA was annealed in a GeneAmp® 9600 thermal cycler using the following thermal profile: 20 sec at 94 °C, 85 °C for 60 sec, 85 °C to 55 °C ramp over 30 min, hold at 55 °C for 15 min. The DNA was transferred to ice after the program finished. The extension/ligation was carried out by adding 8 μl of 10 x synthesis buffer (5 mM each dNTP, 10 mM ATP, 100 mM Tris pH 7.4, 50 mM $MgCl_2$, 20 mM DTT), 8 μl T4 DNA ligase (1 U/μl, Roche Molecular Biochemicals, Indianapolis, IN), 8 μl diluted T7 DNA polymerase (1 U/μl, New England BioLabs, Beverly, MA) and incubating at 37 °C for 30 min. The reaction was stopped with 296 μl of mutagenesis stop buffer (10 mM Tris pH 8.0, 10 mM EDTA). The mutagenesis DNA was extracted once with equilibrated phenol (pH>8):chloroform:isoamyl alcohol (50:49:1), once with chloroform:isoamyl alcohol (49:1), and the DNA was ethanol precipitated at -20 °C for at least 30 min. The DNA was pelleted and the supernatant carefully removed as described above. The sample was briefly spun again and all traces of ethanol removed with a pipetman. The pellet was dried in vacuo. The DNA was resuspended in 4 μl of sterile water. 1 μl mutagenesis DNA was (500 ng) was transferred into 40μl electrocompetent *E. coli* DH12S (Gibco/BRL, Gaithersburg, MD) using the electroporation conditions in Example 8. The transformed cells were mixed with 1.0 mL 2 x YT broth (Sambrook, *et al., supra*) and transferred to a 15 mL sterile culture tube. Aliquots (10μL of 10^{-3} and 10^{-4} dilutions) of the transformed cells were plated on 100mm LB agar plates as described in Example11. After 6 hr of growth at 37°C, 20 individual plaques were picked from a plate into 2.75mL 2 x YT and 0.25ml overnight XL1 blue cells. The cultures were grown at 37°C, 300 rpm overnight to amplify the phage from the individual plaques. The phage samples were analyzed for insertion of both constant regions by PCR using oligonucleotides 197 and 5 (see

above in BS46 analysis), followed by sizing of the PCR products by agarose gel electrophoresis. The sequence of two clones having what appeared to be two inserts by agarose gel electrophoresis was verified at MacConnell Research (San Diego, CA) by the dideoxy chain termination method using a Sequatherm sequencing kit (Epicenter Technologies, Madison, WI) and a LI-COR 4000L automated sequencer (LI-COR, Lincoln, NE). Oligonucleotide primers 885 and 5, that bind on the 3' side of the kappa chain and heavy chain respectively, were used. Both clones had the correct sequence. The uracil template having human constant region sequences, called BS47, was prepared as described in Example 6.

Primer 885
5' TAA GAG CGG TAA GAG TGC CAG

Example 6: Preparation of uracil templates used in generation of spleen antibody phage libraries

1 mL of *E. coli* CJ236 (BioRAD, Hercules, CA) overnight culture and 10μL of a 1/100 dilution of vector phage stock was added to 50 ml 2 x YT in a 250 mL baffled shake flask. The culture was grown at 37 °C for 6 hr. Approximately 40 mL of the culture was centrifuged at 12,000 rpm for 15 minutes at 4 °C. The supernatant (30 mL) was transferred to a fresh centrifuge tube and incubated at room temperature for 15 minutes after the addition of 15 μl of 10 mg/ml RnaseA (Boehringer Mannheim, Indianapolis, IN). The phage were precipitated by the addition of 7.5 ml of 20% polyethylene glycol 8000 (Fisher Scientific, Pittsburgh, PA)/3.5M ammonium acetate (Sigma Chemical Co., St. Louis, MO) and incubation on ice for 30 min. The sample was centrifuged at 12,000 rpm for 15 min at 2-8 °C. The supernatant was carefully discarded, and the tube was briefly spun to remove all traces of supernatant. The pellet was resuspended in 400 μl of high salt buffer (300 mM NaCl, 100 mM Tris pH 8.0, 1 mM EDTA), and transferred to a 1.5 mL tube. The phage stock was extracted repeatedly with an equal volume of equilibrated phenol:chloroform:isoamyl alcohol (50:49:1) until no trace of a white interface was visible, and then extracted with an equal volume of chloroform:isoamyl alcohol (49:1). The DNA was precipitated with 2.5 volumes of ethanol and 1/5 volume 7.5 M ammonium acetate and incubated 30 min at -20 °C. The DNA was centrifuged at 14,000 rpm for 10 min at 4 °C, the pellet washed once with cold 70% ethanol, and dried *in vacuo*. The uracil template DNA was dissolved in 100 μl sterile water and the concentration determined by A_{260} using

an absorbance of 1.0 for a concentration of 40 μg/ml. The template was diluted to 250 ng/μl with sterile water, aliquoted, and stored at -20 °C.

Example 7: Mutagenesis of uracil template with ss-DNA and electroporation into *E. coli* to generate antibody phage libraries

Antibody phage-display libraries were generated by simultaneously introducing single-stranded heavy and light chain genes onto a phage-display vector uracil template. A typical mutagenesis was performed on a 2 μg scale by mixing the following in a 0.2 mL PCR reaction tube: 8 μl of (250 ng/μl) BS47 uracil template (examples 5 and 6), 8 μl of 10 x annealing buffer (200 mM Tris pH 7.0, 20 mM $MgCl_2$, 500 mM NaCl), 3.33 μl of kinased single-stranded heavy chain insert (100 ng/μl) , 3.1 μl of kinased single-stranded light chain insert (100 ng/ml), and sterile water to 80 μl. DNA was annealed in a GeneAmp® 9600 thermal cycler using the following thermal profile: 20 sec at 94 °C, 85 °C for 60 sec, 85 °C to 55 °C ramp over 30 min, hold at 55 °C for 15 min. The DNA was transferred to ice after the program finished. The extension/ligation was carried out by adding 8 μl of 10 x synthesis buffer (5 mM each dNTP, 10 mM ATP, 100 mM Tris pH 7.4, 50 mM $MgCl_2$, 20 mM DTT), 8 μl T4 DNA ligase (1 U/μl), 8 μl diluted T7 DNA polymerase (1 U/μl) and incubating at 37 °C for 30 min. The reaction was stopped with 300 μl of mutagenesis stop buffer (10 mM Tris pH 8.0, 10 mM EDTA). The mutagenesis DNA was extracted once with equilibrated phenol (pH>8):chloroform:isoamyl alcohol (50:49:1), once with chloroform:isoamyl alcohol (49:1), and the DNA was ethanol precipitated at -20 °C for at least 30 min. The DNA was pelleted and the supernatant carefully removed as described above. The sample was briefly spun again and all traces of ethanol removed with a pipetman. The pellet was dried in vacuo. The DNA was resuspended in 4 μl of sterile water. 1 μl mutagenesis DNA was (500 ng) was transferred into 40μl electrocompetent *E. coli* DH12S (Gibco/BRL, Gaithersburg, MD) using the electroporation conditions in Example 8. The transformed cells were mixed with 0.4 mL 2 x YT broth (Sambrook, *et al., supra*) and 0.6mL overnight XL1 Blue cells, and transferred to 15 mL sterile culture tubes. The first round antibody phage samples were generated by plating the electroporated samples on 150mm LB plates as described in Example 11. The plates were incubated at 37°C for 4hr, then 20°C overnight. The first round antibody phage was eluted from the 150 mm plates

by pipeting 10 mL 2YT media onto the lawn and gently shaking the plate at room temperature for 20 min. The phage were transferred to 15 mL disposable sterile centrifuge tubes with plug seal cap and the debris from the LB plate was pelleted by centrifuging for 15 min at 3500 rpm. The 1st round antibody phage was then transferred to a new tube.

The efficiency of the electroporation was measured by plating 10 µl of 10^{-3} and 10^{-4} dilutions of the cultures on LB agar plates (see Example 11). These plates were incubated overnight at 37 °C. The efficiency was determined by multiplying the number of plaques on the 10^{-3} dilution plate by 10^{5} or multiplying the number of plaques on the 10^{-4} dilution plate by 10^{6}.

Example 8: Transformation of E. coli by electroporation

The electrocompetent *E. coli* cells were thawed on ice. DNA was mixed with 20-40 µL electrocompetant cells by gently pipetting the cells up and down 2-3 times, being careful not to introduce air-bubble. The cells were transferred to a Gene Pulser cuvette (0.2 cm gap, BioRAD, Hercules, CA) that had been cooled on ice, again being careful not to introduce an air-bubble in the transfer. The cuvette was placed in the *E. coli* Pulser (BioRAD, Hercules, CA) and electroporated with the voltage set at 1.88 kV according to the manufacturer's recommendation. The transformed sample was immediately diluted to 1 ml with 2 x YT broth or 1ml of a mixture of 400µL 2 x YT/600µL overnight XL1 Blue cells and processed as procedures dictate.

Example 9: Preparation of biotinylated interleukin 8 (IL8)

IL8 was dialyzed against a minimum of 100 volumes of 20 mM borate, 150 mM NaCl, pH 8 (BBS) at 2-8 °C for at least 4 hr. The buffer was changed at least once prior to biotinylation. IL8 was reacted with biotin-XX-NHS ester (Molecular Probes, Eugene, OR, stock solution at 40 mM in dimethylformamide) at a final concentration of 1 mM for 1 hr at room temperature. After 1 hr, the IL8 was extensively dialyzed into BBS to remove unreacted small molecules.

Example 10: Preparation of avidin magnetic latex

The magnetic latex (superparamagnetic microparticles, 0.96 µm, Estapor, 10% solids, Bangs Laboratories, Carmel, IN) was thoroughly resuspended

and 2 ml aliquoted into a 15 ml conical tube. The magnetic latex was suspended in 12 ml distilled water and separated from the solution for 10 min using a magnet. While still in the magnet, the liquid was carefully removed with a 10 mL sterile pipet. This washing process was repeated an additional three times. After the final wash, the latex was resuspended in 2 ml of distilled water. In a separate 50 ml conical tube, 10 mg of avidin-HS (NeutrAvidin, Pierce, Rockford, IL) was dissolved in 18 ml of 40 mM Tris, 0.15 M sodium chloride, pH 7.5 (TBS). While vortexing, the 2 ml of washed magnetic latex was added to the diluted avidin-HS and the mixture vortexed an additional 30 seconds. This mixture was incubated at 45 °C for 2 hr, shaking every 30 minutes. The avidin magnetic latex was separated from the solution using a magnet and washed three times with 20 ml BBS as described above. After the final wash, the latex was resuspended in 10 ml BBS and stored at 4 °C.

Immediately prior to use, the avidin magnetic latex was equilibrated in panning buffer (40 mM TRIS, 150 mM NaCl, 20 mg/mL BSA, 0.1% Tween 20 (Fisher Scientific, Pittsburgh, PA), pH 7.5). The avidin magnetic latex needed for a panning experiment (200µl/sample) was added to a sterile 15 ml centrifuge tube and brought to 10 ml with panning buffer. The tube was placed on the magnet for 10 min to separate the latex. The solution was carefully removed with a 10 mL sterile pipet as described above. The magnetic latex was resuspended in 10 mL of panning buffer to begin the second wash. The magnetic latex was washed a total of 3 times with panning buffer. After the final wash, the latex was resuspended in panning buffer to the initial aliquot volume.

Example 11: Plating M13 phage or cells transformed with antibody phage-display vector mutagenesis reaction

The phage samples were added to 200 µL of an overnight culture of *E. coli* XL1-Blue when plating on 100 mm LB agar plates or to 600 µL of overnight cells when plating on 150 mm plates in sterile 15 ml culture tubes. The electroporated phage samples were in 1mL 2 x YT/overnight XL1 cells, as described in Example 8, prior to plating on 150mm plates. After adding LB top agar (3 mL for 100 mm plates or 9 mL for 150 mm plates, top agar stored at 55 °C, Appendix A1, Molecular Cloning, A Laboratory Manual, (1989) Sambrook. J), the mixture was evenly distributed on an LB agar plate that had been pre-warmed (37 °C-55 °C) to remove

any excess moisture on the agar surface. The plates were cooled at room temperature until the top agar solidified. The plates were inverted and incubated at 37 °C as indicated.

Example 12: Develop nitrocellulose filters with alkaline phosphatase (AP) conjugates

After overnight incubation of the nitrocellulose filters on LB agar plates, the filters were carefully removed from the plates with membrane forceps and incubated for 2 hr in block (1% bovine serum albumin (from 30% BSA, Bayer, Kankakee, IL), 10 mM Tris, 150 mM NaCl, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 0.1% polyvinyl alcohol (80% hydrolyzed, Aldrich Chemical Co., Milwaukee, WI), pH 8.0).

After 2 hr, the filters were incubated with goat anti-human kappa AP (Southern Biotechnology Associates, Inc, Birmingham, AL) for 2-4 hr. The AP conjugate was diluted into block at a final concentration of 1 μg/mL. Filters were washed 3 times with 40 mM TRIS, 150 mM NaCl, 0.05% Tween 20, pH 7.5 (TBST) (Fisher Chemical, Pittsburgh, PA) for 5 min each. After the final wash, the filters were developed in a solution containing 0.2 M 2-amino-2-methyl-1-propanol (JBL Scientific, San Luis Obispo, CA), 0.5 M TRIS, 0.33 mg/mL nitro blue tetrazolium (Fisher Scientific, Pittsburgh, PA) and 0.166 mg/mL 5-bromo-4-chloro-3-indolyl-phosphate, p-toluidine salt.

Example 13: Enrichment of polyclonal phage to Human Interleukin-8 using a decapeptide tag on the kappa chain

The first round antibody phage was prepared as described in Example 7 using BS47 uracil template, which has a decapeptide tag for polyvalent enrichment fused to the kappa chain. Fourteen electroporations of mutagenesis DNA were done from 7 different spleens (2 electroporations from each spleen) yielding 14 different phage samples. Prior to functional panning, the antibody phage samples were enriched for polyvalent display using the decapeptide tag on the kappa chain and the 7F11 magnetic latex. Binding studies had previously shown that the decapeptide could be eluted from the monoclonal antibody 7F11 (see Example 17) at a relatively mild pH of 10.5-11. The 7F11 magnetic latex (2.9 mL) was equilibrated with panning buffer as described above for the avidin magnetic latex (Example 10). Each first round phage stock (1 mL) was aliquoted into a 15 mL tube. The 7F11 magnetic latex

(200 μL per phage sample) was incubated with phage for 10 min at room temperature. After 10 min, 9 mL of panning buffer was added, and the magnetic latex was separated from unbound phage by placing the tubes in a magnet for 10 min. After 10 min in the magnet, the unbound phage was carefully removed with a 10 mL sterile pipet. The magnetic latex was then resuspended in 1mL panning buffer and transferred to 1.5 mL tubes. The magnetic latex was separated from unbound phage by placing the tubes in a smaller magnet for 5 min, then the supernatant was carefully removed with a sterile pipet. The latexes were washed with 1 additional 1 mL panning buffer wash. Each latex was resuspended in 1 mL elution buffer (20 mM 3-(cyclohexylamino)propanesulfonic acid (United States Biochemical, Cleveland, OH), 150 mM NaCl, 20 mg/mL BSA, pH 10.5) and incubated at room temperature for 10 min. After 10 min, tubes were placed in the small magnet again for 5 min and the eluted phage was transferred to a new 1.5 mL tube. The phage samples were again placed in the magnet for 5 min to remove the last bit of latex that was transferred. Eluted phage was carefully removed into a new tube and 25 μL 3 M Tris, pH 6.8 was added to neutralize the phage. Panning with IL8-biotin was set up for each sample by mixing 900 μL 7F11/decapeptide enriched phage, 100 μL panning buffer, and 10 μL 10^{-7} M IL8-biotin and incubating overnight at 2-8 °C.

The antibody phage samples were panned with avidin magnetic latex. The equilibrated avidin magnetic latex (see Example 10), 200 μL latex per sample, was incubated with the phage for 10 min at room temperature. After 10 min, approximately 9 mL of panning buffer was added to each phage sample, and the magnetic latex was washed as described above for the 7F11 magnetic latex. A total of one 9mL and three 1mL panning buffer washes were done. After the last wash, each latex was resuspended in 200μL 2 X YT, then the entire latex of each sample was plated on 150mm LB plates to generate the 2nd round antibody phage. The 150mm plates were incubated at 37°C for 4hr, then overnight at 20°C.

The resulting 2^{nd} round antibody phage samples were set up for the second round of functional panning in separate 15mL disposable sterile centrifuge tubes with plug seal cap by mixing 900 μL panning buffer, 100 μL 2^{nd} round antibody phage, and 10 μL 10^{-7}M interleukin-8-biotin. After overnight incubation at 2-8°C, the phage samples were panned with avidin magnetic latex as described above. Aliquots of one sample from each spleen were plated on 100mm LB agar plates to determine

the percentage of kappa positives (Example 12). The percentage of kappa positives for the 2nd round of panning was between 83-92% for 13 samples. One sample was discarded because it was 63% kappa positive.

The remaining thirteen samples were set up for a third round of functional panning as described above using 950 μL panning buffer, 50 μL 3rd round antibody phage, and 10 μL 10^{-6}M interleukin-8-biotin. After incubation for 1.5 hours at 2-8°C, the phage samples were panned with avidin magnetic latex, and nitrocellulose filters were placed on each phage sample, as described above. The percentage of kappa positives for the 4th round antibody phage samples was estimated to be greater than 80%.

The 4th round antibody phage samples were titered by plating 50μL 10^{-8} dilutions on 100mm LB plates. After 6hr at 37°C, the number of plaques on each plate were counted, and the titers were calculated by multipying the number of plaques by $2x10^9$. A pool of 13-4th round phage was made by mixing an equal number of phage from each phage stock so that high titer phage stocks would not bias the pool. The pooled antibody phage was set up in duplicate for a 4th round of functional panning as described above using 950 μL panning buffer, 50 μL 4th round pooled-antibody phage. One sample (foreground) received 10 μL 10^{-6}M interleukin-8-biotin and the other sample (background) did not receive interleukin-8-biotin and served as a blank to monitor non-specific binding of phage to the magnetic latex. After incubation for 1.5 hours at 2-8°C, the phage samples were panned with avidin magnetic latex as described above. The next day, the 5th round antibody phage was eluted and the number of plaques was counted on the foreground and background plates. The foreground:background ratio was 58:1.

The 5th round antibody phage was set up in triplicate as described above using 950 μL panning buffer, 50 μL 5th round antibody phage per sample with the experimental (foreground) tubes receiving 10 μL 10^{-7}M interleukin-8-biotin or 10 μL 10^{-8}M interleukin-8-biotin, respectively. The third tube did not receive any interleukin-8-biotin. This round of panning or affinity selection preferentially selects for antibodies of $\geq 10^9$ affinity and $\geq 10^{10}$ affinity by including the interleukin-8-biotin at a final concentration of 10^{-9} M and 10^{-10} M, respectively. After greater than 24 hours at 2-8°C, the phage samples were panned with avidin magnetic latex and processed as described above. The 6th round antibody phage sample 10^{-9} M cut had a

foreground:background ratio 1018:1and the 10^{-10}M cut had a foreground:background ratio 225:1.

An additional round of panning was done on the 6th round 10^{-10} M cut antibody phage to increase the number of antibodies with affinity of 10^{10}. The 6th round phage were set up as described above using 975 µL panning buffer, 25 µL 6th round antibody phage per sample with the experimental (foreground) tube receiving 10 µL 10^{-8}M interleukin-8-biotin. The blank did not receive any interleukin-8-biotin. After overnight incubation at 2-8°C, the phage samples were panned with avidin magnetic latex and processed as described above. The 7th round antibody phage sample 10^{-10} M cut had a foreground:background ratio 276:1. The antibody phage populations were subcloned into the expression vector and electroporated as described in Example15.

Example 14: Construction of the pBR expression vector

An expression vector and a process for the subcloning of monoclonal and polyclonal antibody genes from a phage-display vector has been developed that is efficient, does not substantially bias the polyclonal population, and can select for vector containing an insert capable of restoring antibiotic resistance. The vector is a modified pBR322 plasmid, designated pBRncoH3, that contains an arabinose promoter, ampicillin resistance (beta-lactamase) gene, a partial tetracycline resistance gene, a pelB (pectate lyase) signal sequence, and *NcoI* and *HindIII* restriction sites. (Fig. 3). The pBRncoH3 vector can also be used to clone proteins other than Fabs with a signal sequence. A second vector, pBRnsiH3, has been developed for cloning proteins with or without signal sequences, identical to the vector described above except that the pelB signal sequence is deleted and the *NcoI* restriction site has been replaced with an *NsiI* site.

The araC regulatory gene (including the araBAD promoter) was amplified from *E. coli* K-12 strain NL31-001 (a gift from Dr. Nancy Lee at UCSB) by PCR (Example 3) using Taq DNA polymerase (Boehringer Mannheim, Indianapolis, IN) with primers A and B (Table 3). Primers A and B contain 20 base-pairs of the BS39 vector sequence at their 5'-ends complementary to the 5' side of the lac promoter and the 5' side of the pelB signal sequence, respectively. Primer A includes an *EcoRI* restriction site at its 5'-end used later for ligating the ara insert into the pBR

vector. The araCparaBAD PCR product was verified by agarose gel electrophoresis and used as template for an asymmetric PCR reaction with primer 'B' in order to generate the anti-sense strand of the insert. The single-stranded product was run on agarose gel electrophoresis, excised, purified with GeneClean (Bio101, San Diego, CA), and resuspended in water as per manufacturers recommendations. The insert was kinased with T4 polynucleotide kinase for 45 min at 37 °C. The T4 polynucleotide kinase was heat inactivated at 70 °C for 10 min and the insert extracted with an equal volume of phenol/chloroform, followed by chloroform. The DNA was precipitated with ethanol at -20 °C for 30 min. The DNA was pelleted by centrifugation at 14 krpm for 15 min at 4 °C, washed with ice-cold 70% ethanol, and dried *in vacuo*.

The insert was resuspended in water and the concentration determined by A_{260} using an absorbance of 1.0 for a concentration of 40 g/ml. The insert was cloned into the phage-display vector BS39 for sequence verification and to introduce the pelB signal sequence in frame with the arabinose promoter (the pelB signal sequence also contains a *NcoI* restriction site at its 3'-end used later for ligating the ara insert into the pBR vector). The cloning was accomplished by mixing 250 ng of BS39 uracil template (Example 5), 150 ng of kinased araCpBAD insert, and 1.0 l of 10 x annealing buffer in a final volume of 10 l. The sample was heated to 70 C for 2 min and cooled over 20 min to room temperature to allow the insert and vector to anneal. The insert and vector were ligated together by adding 1 l of 10 x synthesis buffer, 1 l T4 DNA ligase (1U/ l), 1 l T7 DNA polymerase (1 U/ l) and incubating at 37 °C for 30 min. The reaction was stopped with 90 l of stop buffer (10 mM Tris pH 8.0, 10 mM EDTA) and 1 l electroporated (Example 8) into electrocompetent*E. coli* strain, DH10B, (Life Technologies, Gaithersburg, MD).

The transformed cells were diluted to 1.0 ml with 2 x YT broth and 1 l, 10 l, 100 l plated as described in Example 12. Following incubation overnight at 37 °C, individual plaques were picked, amplified by PCR with primers A and B, and checked for full-length insert by agarose gel electrophoresis. Clones with full-length insert were sequenced with primers D, E , F, G (Table 3) and checked against the literature. An insert with the correct DNA sequence was amplified by PCR (Example 3) from BS39 with primers A and C (Figure 4A) and the products run on agarose gel electrophoresis.

Full-length products were excised from the gel and purified as described previously and prepared for cloning by digestion with *EcoRI* and *NcoI*. A pBR lac-based expression vector that expressed a murine Fab was prepared to receive this insert by *EcoRI* and *NcoI* digestion. This digestion excised the lac promoter and the entire coding sequence up to the 5'-end of the heavy chain (C_H1) constant region (Figure 4A).

The insert and vector were mixed (2:1 molar ratio) together with 1 l 10 mM ATP, 1 l (1U/ l) T4 DNA ligase, 1 l 10 x ligase buffer in a final volume of 10 l and ligated overnight at 15 °C. The ligation reaction was diluted to 20 l, and 1 l electroporated into electrocompetent*E. coli* strain, DH10B (Example 8), plated on LB tetracycline (10 g/ml) plates and grown overnight at 37 °C.

Clones were picked and grown overnight in 3 ml LB broth supplemented with tetracycline at 20 g/ml. These clones were tested for the correct insert by PCR amplification (Example 3) with primers A and C, using 1 l of overnight culture as template. Agarose gel electrophoresis of the PCR reactions demonstrated that all clones had the araCparaB insert. The vector (plasmid) was purified from each culture by Wizard miniprep columns (Promega, Madison, WI) following manufacturers recommendations. The new vector contained the araC gene, the araB promoter, the pelB signal sequence, and essentially the entire C_H1 region of the heavy chain (Figure 4B).

The vector was tested for expression by re-introducing the region of the Fab that was removed by *EcoRI* and *NcoI* digestion. The region was amplified by PCR, (Example 3) from a plasmid (20 ng) expressing 14F8 with primers H and I (Table 3). The primers, in addition to having sequence specific to 14F8, contain 20 base-pairs of vector sequence at their 5'-end corresponding to the 3'-end of the pelB signal sequence and the 5'-end of the C_H1 region for cloning purposes. The PCR products were run on agarose gel electrophoresis and full-length products excised from the gel and purified as described previously.

The vector was linearized with *NcoI* and together with the insert, prepared for cloning through the 3' 5' exonuclease activity of T4 DNA polymerase. The insert and *NcoI* digested vector were prepared for T4 exonuclease digestion by aliquoting 1.0 g of each in separate tubes, adding 1.0 l of 10 x restriction endonuclease Buffer A (Boehringer Mannheim, Indianapolis, IN) and bringing the volume to 9.0 l with water. The samples were digested for 5 min at 30 °C with 1 l

(1U/ l) of T4 DNA polymerase. The T4 DNA polymerase was heat inactivated by incubation at 70 °C for 15 min. The samples were cooled, briefly spun, and the digested insert (35ng) and vector (100 ng) mixed together and the volume brought to 10 l with 1 mM $MgCl_2$. The sample was heated to 70 °C for 2 min and cooled over 20 min to room temperature to allow the complementary 5' single-stranded overhangs of the insert and vector resulting from the exonuclease digestion to anneal together (Fig. 5). The annealed DNA (1.5 l) was electroporated (Example 8) into 30 l of electrocompetent E. coli strain DH10B.

The transformed cells were diluted to 1.0 ml with 2 x YT broth and 1 l, 10 l, and 100 l plated on LB agar plates supplemented with tetracycline (10 g/ml) and grown overnight at 37 °C. The following day, two clones were picked and grown overnight in 2 x YT (10 g/ml tetracycline) at 37 °C. To test protein expression driven from the ara promoter, these cultures were diluted 1/50 in 2 x YT(tet) and grown to OD_{600}=1.0 at which point they were each split into two cultures, one of which was induced by the addition of arabinose to a final concentration of 0.2% (W/V). The cultures were grown overnight at room temperature, and assayed for Fab production by ELISA. Both of the induced cultures were producing approximately 20 g/ml Fab. There was no detectable Fab in the uninduced cultures.

Initial efforts to clone polyclonal populations of Fab were hindered by backgrounds of undigested vector ranging from 3-13%. This undigested vector resulted in loss of Fab expressing clones due to the selective advantage non-expressing clones have over Fab expressing clones. A variety of means were tried to eliminate undigested vector from the vector preparations with only partial success; examples including: digesting the vector overnight 37 °C with *NcoI*, extracting, and redigesting the preparation a second time; including spermidine in the *NcoI* digest; including single-stranded binding protein (United States Biochemical, Cleveland, OH) in the *NcoI* digest; preparative gel electrophoresis. It was then noted that there is a *HindIII* restriction site in pBR, 19 base-pairs from the 5'-end of the tetracycline promoter. A vector missing these 19 base-pairs is incapable of supporting growth in the presence of tetracycline, eliminating background due to undigested vector.

The ara-based expression vector was modified to make it tetracycline sensitive in the absence of insert. This was done by digesting the pBRnco vector with *NcoI* and *HindIII* (Boehringer Mannheim, Indianapolis, IN), which removed the entire antibody gene cassette and a portion of the tet promoter (Fig. 4B). The region excised

by *NcoI/HindIII* digestion was replaced with a stuffer fragment of unrelated DNA by ligation as described above. The ligation reaction was diluted to 20 l, and 1 l electroporated (Example 8) into electrocompetent *E. coli* strain DH10B, plated on LB ampicillin (100 g/ml) and incubated at 37 °C.

After overnight incubation, transformants were picked and grown overnight in LB broth supplemented with ampicillin (100 g/ml). The vector (plasmid) was purified from each culture by Wizard miniprep columns following manufacturers recommendations. This modified vector, pBRncoH3, is tet sensitive, but still retains ampicillin resistance for growing preparations of the vector.

The antibody gene inserts were amplified by PCR with primers I and J (Table 3) as described in Example 3; primer J containing the 19 base-pairs of the tet promoter removed by *HindIII* digestion, in addition to 20 base-pairs of vector sequence 3' to the *HindIII* site for annealing. This modified vector was digested with *NcoI/HindIII* and, together with the insert, exonuclease digested and annealed as described previously. The tet resistance is restored only in clones that contain an insert capable of completing the tet promoter. The annealed Fab/vector (1 l) was transformed (Example 8) into 30 l of electrocompetent*E. coli* strain, DH10B.

The transformed cells were diluted to 1.0 ml with 2 x YT broth and 10 l of 10^{-2} and 10^{-3} dilutions plated on LB agar plates supplemented with tetracycline at 10 g/ml to determine the size of the subcloned polyclonal population. This plating also provides and opportunity to pick individual clones from the polyclonal if necessary. The remaining cells were incubated at 37 °C for 1 hr and then diluted 1/100 into 30 ml 2 x YT supplemented with 1% glycerol and 20 g/ml tetracycline and grown overnight at 37 °C. The overnight culture was diluted 1/100 into the same media and grown 8 hr at which time glycerol freezer stocks were made for long term storage at -80 °C.

The new vector eliminates growth bias of clones containing vector only, as compared to clones with insert. This, together with the arabinose promoter which is completely repressed in the absence of arabinose, allows cultures of transformed organisms to be expanded without biasing the polyclonal antibody population for antibodies that are better tolerated by *E. coli* until induction.

A variant of this vector was also constructed to clone any protein with or without a signal sequence. The modified vector has the *NcoI* restriction site and all of the pelB signal-sequence removed. In its place a *NsiI* restriction site was

incorporated such that upon *NsiI* digestion and then T4 digestion, there is single base added, in frame, to the araBAD promoter that becomes the adenosine residue (A) of the ATG initiation codon. The *HindIII* site and restoration of the tetracycline promoter with primer J (Table 3) remains the same as described for the pBRncoH3 vector. Additionally, the T4 exonuclease cloning process is identical to that described above, except that the 5' PCR primer used to amplify the insert contains 20 bp of vector sequence at its 5'-end corresponding to 3'-end of the araBAD promoter rather than the 3'-end of the PelB signal sequence.

Three PCR primers, K, L, and M (Table 3) were used for amplifying the araC regulatory gene (including the araBAD promoter). The 5'-primer, primer K, includes an *EcoRI* restriction site at its 5'-end for ligating the ara insert into the pBR vector. The 3'-end of the insert was amplified using two primers because a single primer would have been too large to synthesize. The inner 3'-primer (L) introduces the *NsiI* restriction site, in frame, with the araBAD promoter, with the outer 3' primer (M) introducing the *HindIII* restriction site that will be used for ligating the insert into the vector.

The PCR reaction was performed as in Example 3 on a 4 x 100 l scale; the reactions containing 100 pmol of 5' primer (K), 1 pmol of the inner 3' primer (L), and 100 pmol of outer 3' primer (M), 10 l 2 mM dNTPs, 0.5 L Taq DNA Polymerase, 10 l 10 x Taq DNA polymerase buffer with $MgCl_2$, and H_2O to 100 L. The araCparaBAD PCR product was precipitated and fractionated by agarose gel electrophoresis and full-length products excised from the gel, purified, resuspended in water, and prepared for cloning by digestion with *EcoRI* and *HindIII* as described earlier. The pBR vector (Life Technologies, Gaithersburg, MD) was prepared to receive this insert by digestion with *EcoRI* and *HindIII* and purification by agarose gel electrophoresis as described above.

The insert and vector were mixed (2:1 molar ratio) together with 1 l 10 mM ATP, 1 l (1 U/ l) T4 DNA ligase, 1 l 10 x ligase buffer in a final volume of 10 l and ligated overnight at 15 °C. The ligation reaction was diluted to 20 l, and 1 l electroporated into electrocompetent*E. coli* strain, DH10B (Example 8), plated on LB tetracycline (10 g/ml) plates and grown overnight at 37 °C. Clones were picked and grown overnight in 3 ml LB broth supplemented with tetracycline.

These clones were tested for the correct insert by PCR amplification (Example 3) with primers K and M, using 1 l of overnight culture as template.

Agarose gel electrophoresis of the PCR reactions demonstrated that all clones had the araCparaB insert. The vector (plasmid) was purified from each culture by Wizard miniprep columns following manufacturers recommendations. The new vector, pBRnsi contained the araC gene, the araBAD promoter, and a *NsiI* restriction site.

The vector was tested for expression by introducing a murine Fab. The region was amplified by PCR (Example 3) from a plasmid (20ng) containing a murine Fab with primers O and N (Table 3). The primers, in addition to having sequence specific to the Fab, contain 20 bp of vector sequence at their 5'-end corresponding to the 3'-end araBAD promoter and the 5'-end of the C_H1 region for cloning purposes. The pBRnsi vector was linearized with *NsiI* and *HindIII*. The vector and the PCR product were run on an agarose gel, and full-length products were excised from the gel and purified as described previously. The vector and insert were digested with T4 DNA polymerase and annealed as described earlier. The annealed DNA (1 l) was electroporated (Example 8) into 30 l of electrocompetent*E. coli* strain DH10B. The transformed cells were diluted to 1.0 ml with 2 x YT broth and 1 l, 10 l, and 100 l plated on LB agar plates supplemented with tetracycline (10 g/ml) and grown overnight at 37 °C.

Nitrocellulose lifts were placed on the placed on the surface of the agar plates for 1 min and processed as described (Section 12.24, Molecular Cloning, A laboratory Manual, (1989) Sambrook. J.). The filters were developed with goat anti-kappa-AP, and a positive (kappa expressing) clone was picked and grown overnight in 2 x YT (10 g/ml tetracycline) at 37 °C. The vector (plasmid) was purified from the culture by Wizard miniprep columns (Promega, Madison, WI) following manufacturers recommendations. The Fab region was excised by *NcoI/HindIII* digestion and replaced with a stuffer fragment of unrelated DNA by ligation as described above. The ligation reaction was diluted to 20 l, and 1 l electroporated (Example 8) into electrocompetent *E. coli* strain DH10B, plated on LB ampicillin (100 g/ml) and incubated at 37 °C. After overnight incubation, transformants were picked and grown overnight in LB broth supplemented with ampicillin (100 g/ml). The vector (plasmid) was purified from each culture by Wizard miniprep columns following manufacturers recommendations. This modified vector, pBRnsiH3, is tet sensitive, but still retains ampicillin resistance for growing preparations of the vector.

Example 15: Subcloning polyclonal Fab populations into expression vectors and electroporation into *Escherichia coli*

The polyclonal IL8 antibody phage form both the 10^9 and 10^{10} affinity cuts (see Example 13) were diluted 1/30 in 2 x YT and 1 µl used as template for PCR amplification of the antibody gene inserts with primers 197 (Example 5) and 970 (see below). PCR (3-100 µL reactions) was performed using a high-fidelity PCR system, Expand (Roche Molecular Biochemicals, Indianapolis, IN) to minimize errors incorporated into the DNA product. Each 100 µl reaction contained 100 pmol of 5' primer 197, 100 pmol of 3' primer 970, 0.7 units of Expand DNA polymerase, 10 µl 2 mM dNTPs, 10 µl 10 x Expand reaction buffer, 1 µl diluted phage stock as template, and water to 100 µl. The reaction was carried out in a Perkin-Elmer thermal cycler (Model 9600) using the following thermal profile: one cycle of denaturation at 94 °C (1 min); ten cycles of denaturation (15 sec, 94 °C), annealing (30 sec, 55 °C), elongation (60 sec, 72 °C); fifteen cycles of denaturation (15 sec, 94 °C), annealing (30 sec, 55 °C), elongation (80 sec plus 20 sec for each additional cycle, 72 °C); elongation (6 min, 72 °C); soak (4 °C, indefinitely). The PCR products were ethanol precipitated, pelleted and dried as described above. The DNA was dissolved in water and fractionated by agarose gel electrophoresis. Only full-length products were excised from the gel, purified, and resuspended in water as described earlier.

Primer 970- 5' GT GAT AAA CTA CCG TA AAG CTT ATC GAT GAT AAG CTG TCA A TTA GTG ATG GTG ATG GTG ATG AGA TTT G

The insert and *NcoI/HindIII* digested pBRncoH3 vector were prepared for T4 exonuclease digestion by adding 1.0 µl of 10 x Buffer A to 1.0 µg of DNA and bringing the final volume to 9 µl with water. The samples were digested for 4 min at 30 °C with 1 µl (1U/µl) of T4 DNA polymerase. The T4 DNA polymerase was heat inactivated by incubation at 70 °C for 10 min. The samples were cooled, briefly spun, and 100ng of the digested antibody gene insert and 1 µl of 10 x annealing buffer were mixed with 100ng of digested vector in a 1.5 mL tube. The volume was brought to 10 µl with water, heated to 70 °C for 2 min and cooled over 20 min to room temperature to allow the insert and vector to anneal. The insert and vector were ligated together by adding 1 µl of 10 x synthesis buffer, 1 µl T4 DNA ligase (1U/µl), 1 µl diluted T7 DNA polymerase (1U/µl) and incubating at 37 °C for 15 min.

The ligated DNA (1µl) was diluted into 2µL of water, then 1µL of the diluted DNA was electroporated (Example 8) into 40 µl of electrocompetent *E. coli* strain, DH10B. The transformed cells were diluted to 1.0 ml with 2 x YT broth and 10µl of 10^{-1}, 10^{-2} and 10^{-3} dilutions plated on LB agar plates supplemented with tetracycline at 10 µg/ml to determine the size of the subcloned polyclonal population. The 10^{9} affinity polyclonal had approximately 6000 different clones, and the 10^{10} affinity polyclonal had approximately 10,000 different clones. The remaining cells were incubated at 37 °C, 300rpm for 1 hr, and then the entire culture was transferred into 50 ml 2 x YT supplemented with 1% glycerol and 20 µg/ml tetracycline and grown overnight at 37 °C. The overnight culture was diluted 1/100 into the same media, grown 8 hr, and glycerol freezer stocks made for long term storage at -80 °C.

Monoclonal antibodies were obtained by picking individual colonies off the LB agar plates supplemented with tetracycline used to measure the subcloning efficiency or from plates streaked with cells from the glycerol freezer stocks. The picks were incubated overnight at 37°C, 300rpm in a shake flask containing 2 X YT media and 10µg/mL tetracyclin. Glycerol freezer stocks were made for each monoclonal for long term storage at –80°C. A total of 15 different colonies were picked off of the 10^{9} affinity cut and analyzed for binding to IL8. Of those 15 clones, two expressed a very low amount of antibody, one expressed antibody but did not bind IL8, two expressed functional antibody but the DNA sequence was ambiguous most likely due to sequence template quality, and one expressed functional protein but was not sequenced. Nine clones were sequenced as described in Example 22. A total of 21 different colonies were picked off of the 10^{10} affinity cut and analyzed for binding to IL8. Of those 21 clones, four expressed a very low amount of antibody, three expressed antibody but did not bind IL8, and four expressed functional protein but were not sequenced. Ten clones were sequenced as described in Example 22.

Example 16: Expression of IL8 or Antibodies in Shake Flasks and Purification

A shake flask inoculum is generated overnight from a -80 °C cell bank or from a colony (Example 15) in an incubator shaker set at 37 °C, 300 rpm. The cells are cultured in a defined medium described above. The inoculum is used to seed a 2 L Tunair shake flask (Shelton Scientific, Shelton, CT) which is grown at 37 °C,

300 rpm. Expression is induced by addition of L(+)-arabinose to 2 g/L during the logarithmic growth phase, following which, the flask is maintained at 23 °C, 300 rpm. Following batch termination, the culture is passed through an M-110Y Microfluidizer (Microfluidics, Newton, MA) at 17000 psi.

Purification employs immobilized metal affinity chromatography. Chelating Sepharose FastFlow resin (Pharmacia, Piscataway, NJ) is charged with 0.1 M $NiCl_2$ and equilibrated in 20 mM borate, 150 mM NaCl, 10 mM imidazole, 0.01 % NaN_3, pH 8.0 buffer. A stock solution is used to bring the culture to 10 mM imidazole. The supernatant is then mixed with the resin and incubated for at least 1 hour in the incubator shaker set at room temperature, 150-200 rpm. IL8 or antibody is captured by means of the high affinity interaction between nickel and the hexahistidine tag on the protein. After the batch binding is complete, the resin is allowed to settle to the bottom of the bottle for at least 10 min. The culture is carefully poured out of the bottle, making sure that the resin is not lost. The remaining culture and resin mixture is poured into a chromatography column. After washing, the protein is eluted with 20 mM borate, 150 mM NaCl, 200 mM imidazole, 0.01 % NaN_3, pH 8.0 buffer. If needed, the protein pool is concentrated in a Centriprep-10 concentrator (Amicon, Beverly, MA) at 3500rpm. It is then dialyzed overnight into 20 mM borate, 150 mM NaCl, 0.01 % NaN_3, pH 8.0 for storage, using 12-14,000 MWCO dialysis tubing.

IL8 was further purified by the following procedure. The protein was dialyzed exhaustively against 10mM sodium phosphate, 150mM sodium chloride, pH 7.35, and diluted 1:3 with 10mM sodium phosphate, pH 7.35. This material was loaded onto a Q-Sepharose column (Amersham Pharmacia Biotech, Piscataway, NJ) equilibrated in 10mM sodium phosphate, 40mM NaCl. The IL8 was contained in the flow through fraction. By SDS-polyacrylamide gel analysis, the IL8 was greater than 95% pure. The IL8 was brought to 120mM NaCl and 0.01% NaN_3 and stored at –80°C.

Example 17: Preparation of 7F11 monoclonal antibody

Synthesis of Acetylthiopropionic Acid

To a stirred solution of 3-mercaptopropionic acid (7 ml, 0.08 moles) and imidazole (5.4 g, 0.08 moles) in tetrahydrofuran (THF, 700 ml) was added dropwise over 15 min, under argon, a solution of 1-acetylimidazole (9.6 g, 0.087

moles) in THF (100 ml). The solution was allowed to stir a further 3 hr at room temperature after which time the THF was removed *in vacuo*. The residue was treated with ice-cold water (18 ml) and the resulting solution acidified with ice-cold concentrated HCl (14.5 ml) to pH 1.5-2. The mixture was extracted with water (2 X 50 ml), dried over magnesium sulfate and evaporated. The residual crude yellow oily solid product (10.5 g) was recrystallized from chloroform-hexane to afford 4.8 g (41% yield) acetylthiopropionic acid as a white solid with a melting point of 44-45 °C.

Decapeptide Derivatives

The decapeptide, YPYDVPDYAS, (Chiron Mimotopes Peptide Systems, San Diego, CA) was dissolved (0.3 g) in dry DMF (5.4 mL) in a round bottom flask under argon with moderate stirring. Imidazole (0.02 g) was added to the stirring solution. Separately, acetylthiopropionic acid (0.041 g) was dissolved in 0.55 mL of dry DMF in a round bottom flask with stirring and 0.056 g of 1,1'-carbonyldiimidazole (Aldrich Chemical Co., Milwaukee, WI) was added to the stirring solution. The flask was sealed under argon and stirred for at least 30 min at room temperature. This solution was added to the decapeptide solution and the reaction mixture was stirred for at least six hr at room temperature before the solvent was removed *in vacuo*. The residue in the flask was triturated twice using 10 mL of diethyl ether each time and the ether was decanted. Methylene chloride (20 mL) was added to the residue in the flask and the solid was scraped from the flask and filtered using a fine fritted Buchner funnel. The solid was washed with an additional 20 mL of methylene chloride and the Buchner funnel was dried under vacuum. In order to hydrolyze the derivative to generate a free thiol, it was dissolved in 70% DMF and 1 M potassium hydroxide was added to a final concentration of 0.2 M while mixing vigorously. The derivative solution was allowed to stand for 5 min at room temperature prior to neutralization of the solution by the addition of a solution containing 0.5 M potassium phosphate, 0.1 M borate, pH 7.0, to which concentrated hydrochloric acid has been added to a final concentration of 1 M. The thiol concentration of the hydrolyzed decapeptide derivative was determined by diluting 10 L of the solution into 990 L of a solution containing 0.25 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB, Aldrich Chemical Co., Milwaukee WI) and 0.2 M potassium borate, pH 8.0. The thiol concentration in mM units was equal to the A412(100/13.76).

Preparation of Conjugates of Decapeptide Derivative with Keyhole Limpet Hemocyanin and Bovine Serum Albumin

Keyhole limpet hemocyanin (KLH, 6 ml of 14 mg/ml, Calbiochem, San Diego, CA) was reacted with sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SULFO-SMCC) by adding 15 mg of SULFO-SMCC and maintaining the pH between 7 and 7.5 with 1N potassium hydroxide over a period of one hr at room temperature while stirring. The protein was separated from the unreacted SULFO-SMCC by gel filtration chromatography in 0.1 M potassium phosphate, 0.02 M potassium borate, and 0.15 M sodium chloride, pH 7.0, and 24 ml of KLH-maleimide was collected at a concentration of 3.1 mg/ml. The hydrolyzed decapeptide derivative was separately added to portions of the KLH-maleimide in substantial molar excess over the estimated maleimide amounts present and the solution was stirred for 4 hr at 4 °C and then each was dialyzed against 3 volumes of one liter of pyrogen-free phosphate-buffered saline, pH7.4, prior to immunization.

Bovine serum albumin (BSA, 3.5 ml of 20 mg/ml) was reacted with SMCC by adding a solution of 6.7 mg of SMCC in 0.3 ml acetonitrile and stirring the solution for one hr at room temperature while maintaining the pH between 7 and 7.5 with 1N potassium hydroxide. The protein was separated from unreacted materials by gel filtration chromatography in 0.1 M potassium phosphate, 0.02 M potassium borate, 0.15 M sodium chloride, pH 7.0. The hydrolyzed decapeptide derivative was separately added to portions of the BSA-maleimide in substantial molar excess over the estimated maleimide amounts present and the solution was stirred for 4 hr at 4 °C. The solutions were used to coat microtiter plates for the detection of antibodies that bound to the decapeptide derivative by standard techniques.

Production and Primary Selection of Monoclonal Antibodies

Immunization of Balb/c mice was performed according to the method of Liu, *et al. Clin Chem* **25**:527-538 (1987). Fusions of spleen cells with SP2/0-Ag 14 myeloma cells, propagation of hybridomas, and cloning were performed by standard techniques. Selection of hybridomas for further cloning began with culture

supernatant at the 96-well stage. A standard ELISA procedure was performed with a BSA conjugate of decapeptide derivative adsorbed to the ELISA plate. Typically, a single fusion was plated out in twenty plates and approximately 10-20 wells per plate were positive by the ELISA assay. At this stage, a secondary selection could be performed if antibodies to the SMCC part of the linking arm were to be eliminated from further consideration. An ELISA assay using BSA derivatized with SMCC but not linked to the decapeptide derivative identified which of the positive clones that bound the BSA conjugates were actually binding the SMCC-BSA. The antibodies specific for SMCC-BSA may be eliminated at this step. Monoclonal antibody 7F11, specific for the decapeptide derivative, was produced and selected by this process.

Example 18 Preparation of 7F11 Magnetic Latex

MAG/CM-BSA

To 6 mL of 5 % magnetic latex (MAG/CM, 740 m 5.0 %, Seradyn, Indianapolis, IN) was added 21 mL of water followed by 3 mL of 600 mM 2-(4-morpholino)-ethane sulfonic acid, pH 5.9 (MES, Fisher Scientific, Pittsburgh, PA). Homocysteine thiolactone hydrochloride (HCTL, 480mg, Aldrich Chemical Co., Milwaukee, WI) and 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide (EDAC, 660 mg, Aldrich Chemical Co., Milwaukee, WI) were added in succession, and the reaction mixture was rocked at room temperature for 2 h. The derivatized magnetic latex was washed 3 times with 30 mL of water (with magnet as in Example 14) using probe sonication to resuspend the particles. The washed particles were resuspended in 30 mL of water. Three mL of a solution containing sodium hydroxide (2M) and EDTA (1 mM) was added to the magnetic latex-HCTL suspension, and the reaction proceeded at room temperature for 5 min. The pH was adjusted to 6.9 with 6.45 mL of 1 M hydrochloric acid in 500 mM sodium phosphate, 100 mM sodium borate. The hydrolyzed magnetic latex-HCTL was separated from the supernate with the aid of a magnet, and then resuspended in 33 mL of 50 mM sodium phosphate, 10 mM sodium borate, 0.1 mM EDTA, pH 7.0. The magnetic latex suspension was then added to 2 mL of 36 mg mL-1 BSA-SMCC (made as described in Example 21 with a 5-fold molar excess of SMCC over BSA), and the reaction mixture was rocked overnight at room temperature. N-Hydroxyethylmaleimide (NHEM, 0.42 mL of 500 mM, Organix Inc., Woburn, MA) was added to cap any remaining thiols for 30min. After 30 min, the magnetic latex-BSA was washed twice with 30 mL of 50 mM potassium

phosphate, 10 mM potassium borate, 150 mM sodium chloride, pH 7.0 (50/10/150) and twice with 30 mL of 10 mM potassium phosphate, 2 mM potassium borate, 200 mM sodium thiocyanate, pH 7.0 (10/2/200). The magnetic latex-BSA was resuspended in 30 mL of 10/2/200.

5

7F11-SH (1:5)

To a solution of 7F11 (3.8 mL of 5.85 mg mL^{-1}) was added 18 L of SPDP (40mM in acetonitrile). The reaction proceeded at room temperature for 90 min after which taurine (Aldrich Chemical Co., Milwaukee, WI) was added to a final 10 concentration of 20 mM. Fifteen min later DTT was added to a final concentration of 2 mM, and the reduction reaction proceeded at room temperature for 30 min. The 7F11-SH was purified on G-50 (40 mL) that was eluted with 50/10/150 plus 0.1 mM EDTA. The pool of purified 7F11-SH was reserved for coupling to the MAG/CM-BSA-SMCC.

15

MAG/CM-BSA-7F11

SMCC (10 mg) was dissolved in 0.5 mL of dry dimethylformamide (Aldrich Chemical Co., Milwaukee, WI), and this solution was added to the magnetic latex-BSA suspension. The reaction proceeded at room temperature with gentle 20 rocking for 2 h. Taurine was added to a final concentration of 20 mM. After 20 min the magnetic latex-BSA-SMCC was separated from the supernate with the aid of a magnet and then resuspended in 10/2/200 (20 mL) with probe sonication. The magnetic latex was purified on a column of Superflow-6 (240 mL, Sterogene Bioseparations Inc., Carlsbad, CA) that was eluted with 10/2/200. The buffer was 25 removed, and to the magnetic latex cake was added 30 mL of 0.7 mg mL^{-1} 7F11-SH. The reaction mixture was rocked overnight at room temperature. After 20 hr the reaction was quenched with mercaptoethanol (2 mM, Aldrich Chemical Co., Milwaukee, WI) followed by NHEM (6 mM). The MAG/CM-7F11 was washed with 10/2/200 followed by 50/10/150. The magnetic latex was then resuspended in 30 mL 30 of 50/10/150.

Example 19 Cloning of the mature Human Interleukin-8 antigen

PCR primers A and B (5' and 3' respectively, Table 3) were made corresponding to the coding sequence at the 5'-end of the mature human interleukin-8

antigen and the coding sequence at the 3'-end of human interleukin-8 (Genbank accession number M28130). The 5' primer contains 20 base pairs of vector sequence at its 5'-end corresponding to the 3'-end of the pBRncoH3 vector (Example 14). The 3' primer has six histidine codons inserted between the end of the coding sequence and the stop codon to assist in purification of the recombinant protein by metal-chelate chromatography. The 3' primer also has 19 base-pairs of tet promoter removed from the tet resistance gene in pBRncoH3 by HindIII digestion, and 20 base-pairs of vector sequence 3' to the HindIII site at its 5' end (Example 14).

The PCR amplification of the mature interleukin-8 gene insert was done on a 3x 100 µl reaction scale each containing 100 pmol of 5' primer (A), 100 pmol of 3' primer (B), 2.5 units of Expand polymerase, 10 µl 2 mM dNTPs, 10 µl 10x Expand reaction buffer, 1 µl of Clontech Quick-clone human liver cDNA (Clontech Laboratories, Palo Alto, CA) as template, and water to 100 µl. The reaction was carried out in a Perkin-thermal cycler as described in Example 15. The PCR products were precipitated and fractionated by agarose gel electrophoresis and full-length products excised from the gel, purified, and resuspended in water (Example 14). The insert and NcoI/HindIII digested pBRncoH3 vector were prepared for T4 exonuclease digestion by adding 1.0µl of 10x Buffer A to 1.0µg of DNA and bringing the final volume to 9µl with water. The samples were digested for 4 minutes at 30°C with 1µl (1U/µl) of T4 DNA polymerase. The T4 DNA polymerase was heat inactivated by incubation at 70°C for 10 minutes. The samples were cooled, briefly spun, and 15 ng of the digested insert added to 100 ng of digested pBRncoH3 vector in a fresh microfuge tube. After the addition of 1.0 µl of 10x annealing buffer, the volume was brought to 10 µl with water. The mixture was heated to 70°C for 2 minutes and cooled over 20 minutes to room temperature, allowing the insert and vector to anneal. The annealed DNA was diluted one to four with distilled water and electroporated (example 8) into 30µl of electrocompetent E. *coli* strain, DH10B. The transformed cells were diluted to 1.0ml with 2xYT broth and 10 µl, 100 µl, 300 µl plated on LB agar plates supplemented with tetracycline (10µg/ml) and grown overnight at 37°C. Colonies were picked and grown overnight in 2xYT (20µg/ml tetracycline at 37°C. The following day glycerol freezer stocks were made for long term storage at -80°C. The sequence of these clones was verified at MacConnell Research (San Diego, CA) by the dideoxy chain termination method using a Sequatherm sequencing kit (Epicenter Technologies, Madison, WI), oligonucleotide primers C and D (Table 3) that bind on the 5' and 3' side of the insert

in the pBR vector, respectively, and a LI-COR 4000L automated sequencer (LI-COR, Lincoln, NE).

Table 3: PCR and Sequencing Primer Sequences

A- 5′ (TCGCTGCCCAACCAGCCATGGCCAGTGCTAAAGAACTTAGATCTCAG)
B- 5′ (GTGATAAACTACCGCATTAAAGCTTATCGATGATAAGCTGTCAATTAGTGAT GGTGATGGTGATGTGAATTCTCAGCCCTCTTCAA)
C- 5′ (GCAACTCTCTACTGTTTCTCC)
D- 5′ (GAGGATGACGATGAGCGC)

Example 20. Estimation of Library Diversity

Upon the completion of library selection for a given target antigen, the library contains members encoding antibodies exhibiting an affinity determined by the criteria used during the selection process. Preferably, the selection process is repeated until the majority of the members in the library encode antibodies exhibiting the desired characteristics. Most preferably, the selection process is repeated until substantially all of the members of the library encode antibodies that exhibit the desired affinity for the target antigen. In order to estimate the number of different antibodies in the selected library, individual members are randomly chosen and sequenced to determine if their amino acid sequences are different. Antibodies exhibiting at least one amino acid difference in either the heavy or light chain variable domain (preferably in the CDRs) are considered different antibodies. A random sampling of the library in such a manner provides an estimate of the frequency antibody copies in the library. If ten antibodies are randomly sampled and each antibody amino acid sequence is distinct from the other sampled antibodies, then an estimate of 1/10 can be applied to the frequency that one might expect to observe for repeated antibodies in the library. A library with hundreds or thousands of total members will exhibit a probability distribution for the frequency of antibody copies that closely approximates the Poisson distribution, $Pr(y)= e^{-\eta}\eta^{y}/y!$, where the probability of a particular value y of the frequency is dependent only on the mean frequency η. If no antibody replicates are observed in a random sampling of ten antibodies, then an estimate for η is 0.1 and the probability of not observing a copy of a library member when randomly sampling the library is estimated by $Pr(0)= e^{-0.1}=$

0.9. Multiplying this probability by the total number of members in the library provides an estimate of the total number of different antibodies in the library.

Example 21. Determination of Antibody Affinity for IL-8 Labeled with Biotin

The equilibrium binding constants of individual monoclonal antibodies were determined by analysis of the total and free antibody concentrations after a binding equilibrium was established in the presence of biotinylated IL-8 at 10^{-10} M in a 1% solution of bovine serum albumin buffered at pH 8.0. In all experiments the antibody was mixed with IL-8 and incubated overnight at room temperature before the biotin-labeled IL-8 was removed from the solution by adding superparamagnetic microparticles (0.96 μm, Bangs Laboratories, Carmel, IN) coated with NeutrAvidin™ (deglycosylated avidin, Pierce, Rockford, IL) incubating for 10 minutes, and separating the particles from the solution using a permanent magnet. The supernatant solution was removed from the microtiter wells containing the magnetic particles and the antibody concentration was determined. The concentration of total antibody added to the individual wells was determined by quantifying the antibody in a sample that was not mixed with IL-8. The concentration of immunoreactive antibody (the fraction of the antibody protein that was capable of binding to IL-8) was determined by incubating a large excess of biotin-labeled IL-8 with a known concentration of antibody for a sufficient time to reach equilibrium, removing the IL-8 using magnetic latex as described above, and quantifying the concentration of antibody left in the solution using the assay described below. The fraction of antibody that bound to the excess of IL-8 is the immunoreactive fraction and the fraction that did not bind to IL-8 is the non-immunoreactive fraction. When determining the concentration of total antibody in an equilibrium mixture, the antibody concentration is the amount of total antibody in the mixture determined from the assay described below multiplied by the immunoreactive fraction. Similarly, when calculating the free antibody in an equilibrium mixture after the removal of IL-8, the non-immunoreactive fraction of antibody is subtracted from the free antibody concentration determined by the assay described below. The bound fraction, B, is determined by subtracting the free immunoreactive antibody concentration in the mixture, F, from the total immunoreactive antibody concentration in the mixture. From the Law of Mass

Action, B/F= -KB + KT where T is the total antigen concentration. A plot of B/F vs. B yields a slope of –K and a y-intercept of KT.

To determine the antibody concentrations in samples a sandwich assay was constructed using immobilized monoclonal antibody 7F11 to bind the decapeptide tag present a the C-terminus of the kappa chain and affinity-purified goat-anti-human kappa antibody conjugated to alkaline phosphatase (Southern Biotechnology Associates, Birmingham, Alabama) to bind the kappa chain of each human antibody. A purified antibody of known concentration with the same kappa chain construction as the assayed antibodies was used to calibrate the assay. The 7F11 antibody was labeled with biotin and immobilized on microtiter plates coated with streptavidin using standard methods. The assay was performed by adding 50 µl of sample from the equilibrium mixtures to each well and incubating for four hours at room temperature. The conjugate was added at a final concentration of approximately 0.125 µg/ml to each well and incubated overnight at room temperature. The wells were washed using an automatic plate washer with borate buffered saline containing 0.02% polyoxyethylene 20-sorbitan monolaurate at pH 8.2 and the ELISA Amplification System (Life Technologies, Gaithersburg, MD) was employed to develop the assay. The absorbance at 490 nm was measured using a microtiter plate reader and the unknown antibody concentrations were determined from the standard curve.

Table 4: Affinities of anti-IL-8 antibodies

Monoclonal Antibody	% Immunoreactive Protein	Affinity (10^{10} M^{-1})
M1-3	93	6.1
M1-4	93	22
M1-5	90	11
M1-8	91	10
M1-10	90	6.1
M1-21	67	6.6
M1-23	91	8.9
M1-25	90	6.4
M2-11	93	10
M2-12	93	28
M2-16	90	1.9
M2-18	80	5.4
M2-20	94	37
M2-34	94	27

Example 22. DNA sequence analysis of random clones

The glycerol freezer stocks (Example15) corresponding to each monoclonal Fab to be analyzed were used to inoculate 50ml cultures for plasmid isolation and subsequent DNA sequencing of the interleukin-8 insert. After overnight growth in 2xYT (10μg/ml tetracycline) at 37°C, the recombinant plasmid was purified using a Qiagen Plasmid Midi kit (Qiagen, Valencia, CA) following manufacturer's recommendations. The sequence corresponding to the kappa and heavy chain variable and constant regions for each monoclonal was determined at MacConnell Research (San Diego, CA). The nomenclature used for antibodies is the same as that in Example 21. Sequencing was done by the dideoxy chain termination method using a Sequatherm sequencing kit (Epicenter Technologies, Madison, WI), oligonucleotide primers C and D (Table 3) that bind on the 5' and 3' side of the Fab cassette in the pBR vector, respectively, and a LI-COR 4000L automated sequencer (LI-COR, Lincoln, NE).

M1-1L

AAATTGTGTTGACGCATTCCAGCCACCCTGTCTTTGTCTCCAGGGGAAAGAGCCACCCTCTCCTGCAGG
GCCAGTCAGGGTGTTAGCAGCTACTTAGCCTGGTACCAACAGAAACCTGGCCAGGCTCCCAGGCTCCTC
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TTCACTCTCACCATCAGCAGCCTAGAGCCTGAAGATTTTGCAGTTTATTACTGTCAGCAGCGTAGAACT
GGCCTCGGACGTTCGGCCAAGGGACCAAGGTGGAAATCAAACGAACTGTGGCTGCACCATCTGTCTTCA
TCTTCCCGCCATCTGATGAGCAGTTGAAATCTGGAACTGCCTCTGTTGTGTGCCTGCTGAATAACTTCT
ATCCCAGAGAGGCCAAAGTACAGTGGAAGGTGGATAACGCCCTCCAATCGGGTAACTCCCAGGAGAGTG
TCACAGAGCAGGACAGCAAGGACAGCACCTACAGCCTCAGCAGCACCCTGACGCTGAGCAAAGCAGACT

ACGAGAAACACAAAGTCTACGCCTGCGAAGTCACCCATCAGGGCCTGAGCTCGCCCGTCACAAAGAGCT
TCAACAGGGGAGAGTCTTATCCATATGATGTGCCAGATTATGCGAGC

M1-3L

GAAATAGTGATGACGCAGTCTCCAGCCACCCTGTCTTTGTCTCCAGGGGAAAGAGCCACCCTCTCCTGC
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TCTGTCTTCATCTTCCCGCCATCTGATGAGCAGTTGAAATCTGGAACTGCCTCTGTTGTGTGCCTGCTG
AATAACTTCTATCCCAGAGAGGCCAAAGTACAGTGGAAGGTGGATAACGCCCTCCAATCGGGTAACTCC
CAGGAGAGTGTCACAGAGCAGGACAGCAAGGACAGCACCTACAGCCTCAGCAGCACCCTGACGCTGAGC
AAAGCAGACTACGAGAAACACAAAGTCTACGCCTGCGAAGTCACCCATCAGGGCCTGAGCTCGCCCGTC
ACAAAGAGCTTCAACAGGGGAGAGTCTTATCCATATGATGTGCCAGATTATGCGAGC

M1-4L

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GGTAGCTCATTCACTTTCGGCCCTGGGACCAAAGTGGATATCAAACGAACTGTGGCTGCACCATCTGTC
TTCATCTTCCCGCCATCTGATGAGCAGTTGAAATCTGGAACTGCCTCTGTTGTGTGCCTGCTGAATAAC
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AGTGTCACAGAGCAGGACAGCAAGGACAGCACCTACAGCCTCAGCAGCACCCTGACGCTGAGCAAAGCA
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AGCTTCAACAGGGGAGAGTCTTATCCATATGATGTGCCAGATTATGCGAGC

M1-5L

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AAAGCAGACTACGAGAAACACAAAGTCTACGCCTGCGAAGTCACCCATCAGGGCCTGAGCTCGCCCGTC
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M1-8L

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M1-10L

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AGCTTCAACAGGGGAGAGTCTTATCCATATGATGTGCCAGATTATGCGAGC

M1-21L

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AGTACACCATTCACTTTCGGCCCTGGGACCAAAGTGGATATCAAACGAACTGTGGCTGCACCATCTGTC
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M1-23L

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M1-25L

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M1-1H

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GTGGTGACCGTGCCCTCCAGCAGCTTGGGCACCCAGACCTACATCTGCAACGTGAATCACAAGCCCAGC
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M1-3H

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M1-4H

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M1-5H

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M1-8H

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M1-10H

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M1-21H

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M1-23H

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GCCCTGACCAGCGGCGTGCACACCTTCCCGGCTGTCCTACAGTCCTCAGGACTCTACTCCCTCAGCAGC
GTGGTGACCGTGCCCTCCAGCAGCTTGGGCACCCAGACCTACATCTGCAACGTGAATCACAAGCCCAGC
AACACCAAGGTGGACAAGAAAGCAGAGCCCAAATCTCATCACCATCACCATCAC

M1-25H

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M2-11L

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M2-12L

GAAATAGTGATGACGCAGTCTCCAGGCACCCTGTCTTTGTCTCCAGGGGAAAGAGCCACCCTCTCCTGC
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M2-16L

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M2-18L

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M2-20L

GAAATAGTGATGACGCAGTCTCCAGGCACCCTGTCTTTGTCTCCAGGGGAAAGAGCCACCCTCTCCTGC
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M2-31L

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M2-32L

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M2-33L

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M2-34L

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M2-35L

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M2-11H

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M2-12H

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M2-16H

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M2-18H

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M2-20H

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M2-31H

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M2-32H

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M2-33H

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M2-34H

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M2-35H

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Translated amino acid sequences of sequenced antibodies. M1-H Heavy Chain Variable and CH1 Regions 10-9M Affinity Cut

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       M1_1H  QVQLVESGGG VVQPGKSLRL SCAASEFTIS YYGMHWVRQV PGKGLEWVAA
      M1_21H  QVQLVQSGGG VVQPGKSLRL SCAASGFTFS YYGMHWVRQV PGKGLEWVAA
      M1_23H  QVQLVQSGGG VVQPGRSLRL SCAASGFTFS NYGMHWVRQA PGKGLEWVAA
      M1_25H  QVQLVESGGG LVQPGGSLRL SCAASGFTFS YYGMHWVRQV PGKGLEWVAA
       M1_3H  DVQLVQSGGG VVQPGRSLRL SCAASGFTFS YYGMHWVRQA PGKGLEWVTL
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       M1_5H  QVQLVESGGG VVQPGRSLRL SCAASGFTFS YYGMHWVRQA PGKGLEWVTL
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       M1_1H  VWYDESTTYS PDSVKGRFTI SRDDSKNTLY LQMNSLRAED TAVYYCARDR
      M1_21H  VWYDGSTTYS PDSVKGRFTI SRDDSKNTLY LQMSSLRAED TAVYYCARDR
      M1_23H  IWYDGSKTYN ADSVKGRFTI SRDNSKNTLY LQMNSLRAED TAVYYCARDG
      M1_25H  VWYDGSTTYP PDSVKGRFTI SRDDSKNTLY LQMNSLRAED TAVYYCARDR
       M1_3H  ITYDGDNKYY ADSVKGRFTI SRDNSKNTLY LQMNSLRAED TAVYYCARDG
       M1_4H  VWYDGSTTYS PDSVKGRFTI SRDDSKNTLY LQMNSLRAED TAVYYCARDR
       M1_5H  ITYDGDNKYY ADSVKGRFTI SRDNSKNTLY LQMNSLRAED TAVYYCARDG
       M1_8H  VWYDGSNTYS PDSVKGRFTI SRDDSKNTVY LQMNSLRAED TAVYYCARDR

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       M1_1H  VG........ ....LFDYWG QGTLVTVSSA STKGPSVFPL APSSKSTSGG
      M1_21H  VG........ ....LFDYWG QGTLVTVSSA STKGPSVFPL APSSKSTSGG
      M1_23H  IG........ ....YFDYWG QGTLVTVSSA STKGPSVFPL APSSKSTSGG
      M1_25H  VG........ ....LFDYWG QGTLVTVSSA STKGPSVFPL APSSKSTSGG
       M1_3H  IG........ ....YFDYWG QGTLVTVSSA STKGPSVFPL APSSKSTSGG
       M1_4H  VG........ ....LFDYWG QGTLVTVSSA STKGPSVFPL APSSKSTSGG
       M1_5H  IG........ ....YFDYWG QGTLVTVSSA STKGPSVFPL APSSKSTSGG
       M1_8H  VG........ ....LFDYWG QGTLVTVSSA STKGPSVFPL APSSKSTSGG

                151                                                 200
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       M1_1H  TAALGCLVKD YFPEPVTVSW NSGALTSGVH TFPAVLQSSG LYSLSSVVTV
      M1_21H  TAALGCLVKD YFPEPVTVSW NSGALTSGVH TFPAVLQSSG LYSLSSVVTV
      M1_23H  TAALGCLVKD YFPEPVTVSW NSGALTSGVH TFPAVLQSSG LYSLSSVVTV
      M1_25H  TAALGCLVKD YFPEPVTVSW NSGALTSGVH TFPAVLQSSG LYSLSSVVTV
       M1_3H  TAALGCLVKD YFPEPVTVSW NSGALTSGVH TFPAVLQSSG LYSLSSVVTV
       M1_4H  TAALGCLVKD YFPEPVTVSW NSGALTSGVH TFPAVLQSSG LYSLSSVVTV
       M1_5H  TAALGCLVKD YFPEPVTVSW NSGALTSGVH TFPAVLQSSG LYSLSSVVTV
       M1_8H  TAALGCLVKD YFPEPVTVSW NSGALTSGVH TFPAVLQSSG LYSLSSVVTV

                201                                     237
      M1_10H  PSSSLGTQTY ICNVNHKPSN TKVDKKAEPK SHHHHHH
       M1_1H  PSSSLGTQTY ICNVNHKPSN TKVDKKAEPK SHHHHHH
      M1_21H  PSSSLGTQTY ICNVNHKPSN TKVDKKAEPK SHHHHHH
      M1_23H  PSSSLGTQTY ICNVNHKPSN TKVDKKAEPK SHHHHHH
      M1_25H  PSSSLGTQTY ICNVNHKPSN TKVDKKAEPK SHHHHHH
       M1_3H  PSSSLGTQTY ICNVNHKPSN TKVDKKAEPK SHHHHHH
       M1_4H  PSSSLGTQTY ICNVNHKPSN TKVDKKAGPK SHHHHHH
       M1_5H  PSSSLGTQTY ICNVNHKPSN TKVDKKAEPK SHHHHHH
       M1_8H  PSSSLGTQTY ICNVNHKPSN TKVDKKAEPK SHHHHHH
```

M1-L Kappa Chain Variable and Constant Regions 10-9 Affinity Cut

```
                1                                                   50
      M1_10L  DVVMTQSPAT LSLSPGERAT LSCRASQSVS S.YLAWYQQK PGQAPRLLIY
       M1_1L  EIVLTQSPAT LSLSPGERAT LSCRASQGVS S.YLAWYQQK PGQAPRLLIY
      M1_21L  AIRMTQSPSF LSASVGDRVT ITCRASQSIS S.YLNWYQQK PGKAPKLLIY
      M1_23L  EIVLTQSPGT LSLSPGERAT LSCRASQSVS SSYLAWYQQK PGQAPRLLIY
      M1_25L  EIVLTQSPGT LSLSPGERAT LSCRASQSVS SSYLAWYQQK PGQAPRLLIY
       M1_3L  EIVMTQSPAT LSLSPGERAT LSCRASQSVS SSYLAWYQQK PGQAPRLLIY
       M1_4L  EIVLTQSPGT LSLSPGERAT LSCRASQSVS SSYLAWYQQK PGQAPRLHIY
       M1_5L  EIVMTQSPGT LSLSPGERAT LSCRASQSVS SSYLAWYQQK PGQAPRLLIY
       M1_8L  EIVMTQSPGT LSLSPGERAT LSCRASQSVS STYLAWYQQK PGQAPRLLIY

                51                                                  100
      M1_10L  DASNRATGIP ARFSGSGSGT DFTLTISSLE PEDFAVYYCQ QRSNWP.PTF
       M1_1L  DASNRATGIP ARFSGSGSGT DFTLTISSLE PEDFAVYYCQ QRSNWP.RTF
      M1_21L  AASSLQSGVP SRFSVSGSGT DLTLTISSLQ PEDFATYYCQ CGYSTP.FTF
      M1_23L  GASSRATGIP DRFSGSGSGT DFTLTISRLE PEDFAVYYCQ QYGSSPPYTF
      M1_25L  GASSRATGIP NRFSGSGSGT DFTLTISRLE PEDFAVYYCQ QYGSS..FTF
       M1_3L  GASSRATGIP DRFSGSGSGT DFTLTISRLE PEDFAVYYCQ QYGSSPPFTF
       M1_4L  GASRRATGIP DRFSGSGSGT DFTLTISRLE PEDFAVYYCQ QFGSS..FTF
       M1_5L  GASSRATGIP DRFSGSGSGT DFTLTISRLE PEDFAVYYCQ QYGSSPIFTF
       M1_8L  GASSRATGIP DRFSGSGSGT DFTLTISRLE PEDFAVYYCQ QYVSS..FTF

                101                                                 150
      M1_10L  GGGTKVEIKR TVAAPSVFIF PPSDEQLKSG TASVVCLLNN FYPREAKVQW
       M1_1L  GQGTKVEIKR TVAAPSVFIF PPSDEQLKSG TASVVCLLNN FYPREAKVQW
      M1_21L  GPGTKVDIKR TVAAPSVFIF PPSDEQLKSG TASVVCLLNN FYPREAKVQW
      M1_23L  GQGTKLEIKR TVAAPSVFIF PPSDEQLKSG TASVVCLLNN FYPREAKVQW
      M1_25L  GPGTKVDIKR TVAAPSVFIF PPSDEQLKSG TASVVCLLNN FYPREAKVQW
       M1_3L  GPGTKVDIKR TVAAPSVFIF PPSDEQLKSG TASVVCLLNN FYPREAKVQW
       M1_4L  GPGTKVDIKR TVAAPSVFIF PPSDEQLKSG TASVVCLLNN FYPREAKVQW
       M1_5L  GPGTKVDIKR TVAAPSVFIF PPSDEQLKSG TASVVCLLNN FYPREAKVQW
       M1_8L  GPGTKVDIKR TVAAPSVFIF PPSDEQLKSG TASVVCLLNN FYPREAKVQW

                151                                                 200
      M1_10L  KVDNALQSGN SQESVTEQDS KDSTYSLSST LTLSKADYEK HKVYACEVTH
       M1_1L  KVDNALQSGN SQESVTEQDS KDSTYSLSST LTLSKADYEK HKVYACEVTH
      M1_21L  KVDNALQSGN SQESVTEQDS KDSTYSLSST LTLSKADYEK HKVYACEVTH
      M1_23L  RVDNALQSGN SQESVTEQDS KDSTYSLSST LTLSKADYEK HKVYACEVTH
      M1_25L  KVDNALQSGN SQESVTEQDS KDSTYSLSST LTLSKADYEK HKVYACEVTH
       M1_3L  KVDNALQSGN SQESVTEQDS KDSTYSLSST LTLSKADYEK HKVYACEVTH
       M1_4L  KVDNALQSGN SQESVTEQDS KDSTYSLSST LTLSKADYEK HKVYACEVTH
       M1_5L  KVDNALQSGN SQESVTEQDS KDSTYSLSST LTLSKADYEK HKVYACEVTH
       M1_8L  KVDNALQSGN SQESVTEQDS KDSTYSLSST LTLSKADYEK HKVYACEVTH

                201                    226
      M1_10L  QGLSSPVTKS FNRGESYPYD VPDYAS
       M1_1L  QGLSSPVTKS FNRGESYPYD VPDYAS
      M1_21L  QGLSSPVTKS FNRGESYPYD VPDYAS
      M1_23L  QGLSSPVTKS FNRGESYPYD VPDYAS
      M1_25L  QGLSSPVTKS FNRGESYPYD VPDYAN
       M1_3L  QGLSSPVTKS FNRGESYPYD VPDYAS
       M1_4L  QGLSSPVTKS FNRGESYPYD VPDYAS
       M1_5L  QGLSSPVTKS FNRGESYPYD VPDYAS
       M1_8L  QGLSSPVTKS FNRGESYPYD VPDYAS
M2-H Heavy Chain VH-CH1 Sequence 10-10M Affinity Cut

                1                                                   50
      M2_11H  QVQLVESGGG VVQPGRSLRL SCAASGFTFS YYGMHWVRQA PGKGLEWVTL
      M2_12H  DVQLVESGGG VVHPGRSLRL SCAASGFTFS YYGMHWVRQA PGKGLEWMTL
      M2_16H  QVQLVQSGGG VVQPGKSLRL SCAASGFSLS YYGMHWVRQV PGKGLEWVAA
```

```
          M2_18H  QVQLVQSGGG VVQPGKSLRL SCAASGFSFS YYGMHWVRQV PGKGLEWVAA
          M2_20H  QVQLVQSGGG VVQPGRSLRL SCAASGFTFS YYGMHWVRQA PGKGLEWVSL
          M2_31H  QVQLVESGGV VVQPGRSLRL SCAASGFTFS YYGIHWVRQV PGKGLEWVAL
          M2_32H  QVQLVQSGGG LVHPGGSLRL SCEGSGFIFR NHPIHWVRQA PGKGLEWVSV
          M2_33H  QVQLVQSGGG VVQPGRSLRL SCAASGFTFS YYGMHWVRQA PGKGLEWMTL
          M2_34H  QVQLVESGGG VVQPGRSLRL SCAASGFTFS YYGIHWVRQV PGKGLEWVVL
          M2_35H  QVQLVESGGG VVQPGRSLRL SCAASGFTIS YYGIHWVRQV PGKGLEWVEL

                  51                                                  100
          M2_11H  ITYDGDNKYY ADSVKGRFTI SRDNSKNTLY LQMNSLRAED TAVYYCARDG
          M2_12H  ISYDGDNKYY ADSVKGRFTI SRENSKNTLY LQMNSLRAED TAVYYCARDG
          M2_16H  VWYDGSTRYS PDSVKGRFTI SRDDSKNTLY LQMNSLRAED TAVYYCARDR
          M2_18H  VWYDGSTTYS PDSVKGRFTI SRDDSKNTLY LQMNSLRAED TAVYYCARDR
          M2_20H  ITYDGRNKYY ADSVKGRFTI SRENSKNTLY LQMNSLRTED TAEYYCARDG
          M2_31H  ISYDGSNKYY ADSVKGRFTI SRDNSKNTLY LQMNSLRAED TAVYYCARDW
          M2_32H  SGIGG.DTYY ADSVKGRFSI SRDNAKNSLY LQMNSLRAED MAVYYCAREY
          M2_33H  ITYDGDNKYY ADSVKGRFTI SRDNSKNTLY LQMNSLRAED TAVYYCARDG
          M2_34H  ISYDGSNKYY ADSVKGRFTI SRDNSKNTLY LQMNSLRAED TAVYYCARDW
          M2_35H  ISYDGSNKYY ADSVKGRFTI SRDNSKNTLY LQMNSLRAED TAVYYCARDW

                  101                                                 150
          M2_11H  IG........ ....YFDYWG QGTLVTVSSA STKGPSVFPL APSSKSTSGG
          M2_12H  IG........ ....YFDYWG QGTLVTVSSA STKGPSVFPL APSSKSTSGG
          M2_16H  VG........ ....LFDYWG QGTLVTVSSA STKGPSVFPL APSSKSTSGG
          M2_18H  VG........ ....LFDYWG QGTLVTVSSA STKGPSVFPL APSSKSTSGG
          M2_20H  IG........ ....YFDYWG QGILVTVSSA STKGPSVFPL APSSKSTSGG
          M2_31H  IG........ ....YFDYWG QGTLVTVSSA STKGPSVFPL APSSKSTSGG
          M2_32H  YYGSGSYRVD YYYYGMDVWG QGTTVTVSSA STKGPSVFPL APSSKSTSGG
          M2_33H  IG........ ....YFDYWG QGTLVTVSSA STKGPSVFPL APSSKSTSGG
          M2_34H  IG........ ....YFDYWG QGTLVTVSSA STKGPSVFPL APSSKSTSGG
          M2_35H  IG........ ....YFDYWG QGTLVTVSSA STKGPSVFPL APSSKSTSGG

                  151                                                 200
          M2_11H  TAALGCLVKD YFPEPVTVSW NSGALTSGVH TFPAVLQSSG LYSLSSVVTV
          M2_12H  TAALGCLVKD YFPEPVTVSW NSGALTSGVH TFPAVLQSSG LYSLSSVVTV
          M2_16H  TAALGCLVKD YFPEPVTVSW NSGALTSGVH TFPAVLQSSG LYSLSSVVTV
          M2_18H  TAALGCLVKD YFPEPVTVSW NSGALTSGVH TFPAVLQSSG LYSLSSVVTV
          M2_20H  TAALGCLVKD YFPEPVTVSW KSGALTSGVH TFPAVLQSSG LYSLSSVVTV
          M2_31H  TAALGCLVKD YFPEPVTVSW NSGALTSGVH TFPAVLQSSG LYSLSSVVTV
          M2_32H  TAALGCLVKD YFPEPVTVSW NSGALTSGVH TFPAVLQSSG LYSLSSVVTV
          M2_33H  TAALGCLVKD YFPEPVTVSW NSGALTSGVH TFPAVLQSSG LYSLSSVVTV
          M2_34H  TAALGCLVKD YFPEPVTVSW NSGALTSGVH TFPAVLQSSG LYSLSSVVTV
          M2_35H  TAALGCLVKD YFPEPVTVSW NSGALTSGVH TFPAVLQSSG LYSLSSVVTV

                  201                                     237
          M2_11H  PSSSLGTQTY ICNVNHKPSN TKVDKKAEPK SHHHHHH
          M2_12H  PSSSLGTQTY ICNVNHKPSS TKVDKKAEPK SHHHHHH
          M2_16H  PSSSLGTQTY ICNVNHKPSN TKVDKKAEPK SHHHHHH
          M2_18H  PSSSLGTQTY ICNVNHKPSN TKVDKKAEPK SHHHHHH
          M2_20H  PSSSLGTQTY ICNVNHKPSN TKVDKKAEPK SHHHHHH
          M2_31H  PSSSLGTQTY ICNVNHKPSN TKVDKKAEPK SHHHHHH
          M2_32H  PSSSLGTQTY ICNVNHKPSN TKVDKKAEPK SHHHHHH
          M2_33H  PSSSLGTQTY ICNVNHKPSN TKVDKKAEPK SHHHHHH
          M2_34H  PSSSLGTQTY ICNVNHKPSN TKVDKKAEPK SHHHHHH
          M2_35H  PSSSLGTQTY ICNVNHKPSN TKVDKKAEPK SHHHHHH

M2-L Kappa Chain VKCK 10-10M Affinity Cut (Thu Sep 23)

                  1                                                   50
          M2_11L  EIVMTQSPGT LSLSPGERAT LSCRASQGVS SSYLAWYQQK PGQAPRLLIY
          M2_12L  EIVMTQSPGT LSLSPGERAT LSCRASQGVS SSYLAWYQQK PGQAPRLLIY
```

```
          M2_16L  EIVMTQSPGT LSLSPGERAT LSCRASQSVS SSYLAWYQQK PGQAPRLLIY
          M2_18L  EIVMTQSPGT LSLSPGERAT LSCRASQSVS STYLAWYQQK PGQAPRLLIY
          M2_20L  EIVMTQSPGT LSLSPGERAT LSCRASQSVS SSYLAWYQQK PGQAPRLLIY
          M2_31L  EIVLTQSPAT LSLSPGERAT LSCRASQSVS S.YLAWYQQK PGQAPRLLIY
          M2_32L  EIVLTQSPAT LSLSPGERAT LSCRASQSVS S.YLAWYQQK PGQAPRLLIY
          M2_33L  EIVLTQSPGT LSLSPGERAT LSCRASQSVS SSYLAWYQQK PGQAPRLLIY
          M2_34L  EIVLTQSPAT LSLSPGERAT LSCRASQSVS S.YLAWYQQK PGQAPRLLIY
          M2_35L  EIVLTQSPAT LSLSPGERAT LSCRASQSVS S.YLAWYQQK PGQAPRLLIY

                  51                                                  100
          M2_11L  GASSRATGIP DRFSGSGSGT DFTLTISRLE PEDFAVYYCQ QYGSSPPFTF
          M2_12L  GASSRATGIP DRFSGSGSGT DFTLTISSLE PEDFAVYYCQ QYGSSPPYTF
          M2_16L  GASSRATGIP DRFSVSGSGT DFTLTISRLE PEDFAVYYCQ QYGSS..FTF
          M2_18L  GASSRATGIP DRFSGSGSGT DFTLTISRLE PEDFAVYYCQ QYVSS..FTF
          M2_20L  GASRRATGIP DRFSGSGSGT DFTLTISRLE PEDFAVYYCQ QYGSSPMYTF
          M2_31L  DASNRATGIP ARFSGSGSGT DFTLTISSLE PEDFAVYYCQ QRTNWP.RTF
          M2_32L  DASNRAAGIP ARFSGSGSGT DFTLTISSLE PEDFAVYYCQ QRNNWP.LTF
          M2_33L  GASSRATGIP DRFSGSGSGT DFTLTISRLE PEDFAVYYCQ QYGSSPPYTF
          M2_34L  DASNRATGIP ARFSGSGSGT DFTLTISSLE PEDFAVYYCQ QRTNWP.RTF
          M2_35L  DASNRATGIP ARFSGSGSGT DFTLTISSLE PEDFAVYYCQ QRTNWP.RTF

                  101                                                 150
          M2_11L  GPGTKVDIKR TVAAPSVFIF PPSDEQLRSG TASVVCLLNN FYPREAKVQW
          M2_12L  GQGTKLEIKR TVAAPSVFIF PPSDEQLKSG TASVVCLLNN FYPREAKVQW
          M2_16L  GPGTKVDIKR TVAAPSVFIF PPSDEQLKSG TASVVCLLNN FYPREAKVQW
          M2_18L  GPGTKVDIKR TVAAPSVFIF PPSDEQLKSG TASVVCLLNN FYPREAKVQW
          M2_20L  GQGTKLEIKR TVAAPSVFIF PPSDEQLKSG TASVVCLLNN FYPREAKVQW
          M2_31L  GQGTKVEIKR TVAAPSVFIF PPSDEQLKSG TASVVCLLNN FYPREAKVQW
          M2_32L  GGGTKVEIKR TVAAPSVFIF PPSDEQLKSG TASVVCLLNN FYPREAKVQW
          M2_33L  GQGTKLEIKR TVAAPSVFIF PPSDEQLKSG TASVVCLLNN FYPREAKVQW
          M2_34L  GQGTKVEIKR TVAAPSVFIF PPSDEQLKSG TASVVCLLNN FYPREAKVQW
          M2_35L  GQGTKVEIKR TVAAPSVFIF PPSDEQLKSG TASVVCLLNN FYPREAKVQW

                  151                                                 200
          M2_11L  KVDNALQSGN SQESVTEQDS KDSTYSLSST LTLSKADYEK HKVYACEVTH
          M2_12L  KVDNALQSGN SQESVTEQDS KDSTYSLSST LTLSKADYEK HKVYACEVTH
          M2_16L  KVDNALQSGN SQESVTEQDS KDSTYSLSST LTLSKADYEK HKVYACEVTH
          M2_18L  KVDNALQSGN SQESVTEQDS KDSTYSLSST LTLSKADYEK HKVYACEVTH
          M2_20L  KVDNALQSGN SQESVTEQDS KDSTYSLSST LTLSKADYEK HKVYACEVTH
          M2_31L  KVDNALQSGN SQESVTEQDS KDSTYSLSST LTLSKADYEK HKVYACEVTH
          M2_32L  KVDNALQSGN SQESVTEQDS KDSTYSLSST LTLSKADYEK HKVYACEVTH
          M2_33L  KVDNALQSGN SQESVTEQDS KDSTYSLSST LTLSKADYEK HKVYACEVTH
          M2_34L  KVDNALQSGN SQESVTEQDS KDSTYSLSST LTLSKADYEK HKVYACEVTH
          M2_35L  KVDNALQSGN SQESVTEQDS KDSTYSLSST LTLSKADYEK HKVYACEVTH

                  201                     226
          M2_11L  QGLSSPVTKS FNRGESYPYD VPDYAS
          M2_12L  QGLSSPVTKS FNRGESYPYD VPDYAS
          M2_16L  QGLSSPVTKS FNRGESYPYD VPDYAS
          M2_18L  QGLSSPVTKS FNRGESYPYD VPDYAS
          M2_20L  QGLSSPVTKS FNRGESYPYD VPDYAS
          M2_31L  QGLSSPVTKS FNRGESYPYD VPDYAS
          M2_32L  QGLSSPVTKS FNRGESYPYD VPDYAS
          M2_33L  QGLSSPVTKS FNRGESYPYD VPDYAS
          M2_34L  QGLSSPVTKS FNRGESYPYD VPDYAS
          M2_35L  QGLSSPVTKS FNRGESYPYD VPDYAS
```

Example 23 Cloning of the Human Interleukin-8 antigen into the pEF1 vector

PCR primers A and B (5' and 3' respectively, Table 5) were made corresponding to the coding sequence at the 5'-end of the human interleukin-8 antigen and the coding sequence at the 3'-end of human interleukin-8 (Genbank accession number M28130). The 5' primer also contains 21 base pairs of pEF1/Myc-His (A) vector sequence (Invitrogen, San Diego, CA) at its 5'-end corresponding to the *EcoRI* site and sequence immediately upstream. The 3' primer contains an additional 24 base-pairs of vector sequence, including the *PmeI* site and sequence immediately downstream, at its 5' end. The vector sequence at the 5'- ends of these primers will form, upon treatment with T4 DNA polymerase, single-stranded overhangs that are specific and complementary to those on the vector as described in Example 14.

The PCR amplification of the interleukin-8 gene insert was first done on a 50 µl reaction scale containing 50 pmol of 5' primer (A), 50 pmol of 3' primer (B), 1 unit of Expand polymerase, 5 µl 2 mM dNTPs, 5 µl 10x Expand reaction buffer, 1 µl of Clontech Quick-clone human liver cDNA (Clontech Laboratories, Palo Alto, CA) as template, and water to 50 µl. The reaction was carried out in a Perkin-Elmer thermal cycler as described in Example 15. A second PCR amplification was performed on a 3x 100 µl reaction scale in order to prepare sufficient material for cloning, with each reaction containing 100 pmol of 5' primer (A), 100 pmol of 3' primer (B), 2.5 units of Expand polymerase, 10 µl 2 mM dNTPs, 10 µl 10x Expand reaction buffer, 2 µl of the first PCR reaction as template, and water to 100 µl. The PCR products were precipitated and fractionated by agarose gel electrophoresis and full-length products excised from the gel, purified, and resuspended in water (Example 14). The pEF1/Myc-His (A) vector was prepared to receive insert by digestion with *PmeI* and *EcoRI* (New England BioLabs, Beverly, MA). The insert and *EcoRI/PmeI* digested pEF1/Myc-His (A) vector were prepared for T4 exonuclease digestion by adding 1.0µl of 10x Buffer A to 1.0µg of DNA and bringing the final volume to 9µl with water. The samples were digested for 4 minutes at 30°C with 1µl (1U/µl) of T4 DNA polymerase. The T4 DNA polymerase was heat inactivated by incubation at 70°C for 10 minutes. The samples were cooled, briefly spun, and 20 ng of the digested insert added to 100 ng of digested pEF1/Myc-His (A) vector in a fresh microfuge tube. After the addition of 1.0 µl of 10x annealing buffer, the volume was brought to 10 µl with water. The mixture was heated to 70°C for 2 minutes and cooled

over 20 minutes to room temperature, allowing the insert and vector to anneal. The annealed DNA was diluted one to four with distilled water and electroporated (example 8) into 30µl of electrocompetent E. *coli* strain, DH10B. The transformed cells were diluted to 1.0 ml with 2xYT broth and 10 µl, 100 µl, 300 µl plated on LB agar plates supplemented with ampicillin (75µg/ml) and grown overnight at 37°C. Colonies were picked and grown overnight in 2xYT (75µg/ml ampicillin at 37°C. The following day glycerol freezer stocks were made for long term storage at -80°C. The sequence of these clones (pEF1-IL8) was verified at MacConnell Research (San Diego, CA) by the dideoxy chain termination method using a Sequatherm sequencing kit (Epicenter Technologies, Madison, WI), oligonucleotide primers C and D (Table 5) that bind on the 5' and 3' side of the insert in the pEF1/Myc-His (A) vector, respectively, and a LI-COR 4000L automated sequencer (LI-COR, Lincoln, NE).

Table 5: PCR and Sequencing Primer Sequences

A- 5'(TAGTCCAGTGTGGTGGAATTCGCCACCATGACTTCCAAGCTGGCCGT)
B- 5'(CGAGGCTGATCAGCGGGTTTAAACTTATGAATTCTCAGCCCTCTTCAA)
C- 5'(CATTCTCAAGCCTCAGACAGTGG)
D- 5'(CAGACAATGCGATGCAATTTCC)

Example 24 Cloning of the Human Myelin Proteolipid Protein (PLP) antigen into the pEF1 vector

PCR primers A and B (5' and 3' respectively, Table 6) were made corresponding to the coding sequence at the 5'-end of the human PLP antigen and the coding sequence at the 3'-end of human PLP (Genbank accession number M54927). The 5' primer also contains 21 base pairs of pEF1/Myc-His (A) vector sequence (Invitrogen, San Diego, CA) at its 5'-end corresponding to the *EcoRI* site and sequence immediately upstream. The 3' primer contains an additional 24 base-pairs of vector sequence, including the *PmeI* site and sequence immediately downstream, at its 5' end. The vector sequence at the 5'- ends of these primers will form, upon treatment with T4 DNA polymerase, single-stranded overhangs that are specific and complementary to those on the vector as described in Example 14.

The PCR amplification of the PLP gene insert was done on a 2x 100 µl reaction scale containing 100 pmol of 5' primer (A), 100 pmol of 3' primer (B), 2.5 units of Expand polymerase, 10 µl 2 mM dNTPs, 10 µl 10x Expand reaction buffer, 1 µl of

Clontech Quick-clone human brain cDNA (Clontech Laboratories, Palo Alto, CA) as template, and water to 100 µl. The reaction was carried out in a Perkin-Elmer thermal cycler as described in Example 15. The PCR products were precipitated and fractionated by agarose gel electrophoresis and full-length products excised from the gel, purified, and resuspended in water (Example 14). The pEF1/Myc-His (A) vector was prepared to receive insert by digestion with *PmeI* and *EcoRI* (New England BioLabs, Beverly, MA). The insert and *EcoRI/PmeI* digested pEF1/Myc-His (A) vector were prepared for T4 exonuclease digestion by adding 1.0µl of 10x Buffer A to 1.0µg of DNA and bringing the final volume to 9µl with water. The samples were digested for 4 minutes at 30°C with 1µl (1U/µl) of T4 DNA polymerase. The T4 DNA polymerase was heat inactivated by incubation at 70°C for 10 minutes. The samples were cooled, briefly spun, and 45 ng of the digested insert added to 100 ng of digested pEF1/Myc-His (A) vector in a fresh microfuge tube. After the addition of 1.0 µl of 10x annealing buffer, the volume was brought to 10 µl with water. The mixture was heated to 70°C for 2 minutes and cooled over 20 minutes to room temperature, allowing the insert and vector to anneal. The annealed DNA was diluted one to four with distilled water and electroporated (example 8) into 30µl of electrocompetent E. *coli* strain, DH10B. The transformed cells were diluted to 1.0 ml with 2xYT broth and 10 µl, 100 µl, 300 µl plated on LB agar plates supplemented with ampicillin (75µg/ml) and grown overnight at 37°C. Colonies were picked and grown overnight in 2xYT (75µg/ml ampicillin at 37°C. The following day glycerol freezer stocks were made for long term storage at -80°C. The sequence of these clones (pEF1-PLP) was verified at MacConnell Research (San Diego, CA) by the dideoxy chain termination method using a Sequatherm sequencing kit (Epicenter Technologies, Madison, WI), oligonucleotide primers C and D (Table 6) that bind on the 5' and 3' side of the insert in the pEF1/Myc-His (A) vector, respectively, and a LI-COR 4000L automated sequencer (LI-COR, Lincoln, NE).

Table 6: PCR and Sequencing Primer Sequences

A- 5'(TAGTCCAGTGTGGTGGAATTCGCCACCATGGGCTTGTTAGAGTGCTGTG)
B- 5'(CGAGGCTGATCAGCGGGTTTAAACTCAGAACTTGGTGCCTCGGCCCAT)
C- 5'(CATTCTCAAGCCTCAGACAGTGG)
D- 5'(CAGACAATGCGATGCAATTTCC)

Example 25 Purification of pEF1-IL8 and pEF1-PLP plasmids and stable transfection of PLP in COS-7 cells

A single colony of *E. coli* containing pEF1-IL8 (Example 23) or pEF1-PLP (Example 24) plasmid was cultured in LB containing 50 μg/ml of ampicillin for 8h at 37°C with shaking at 300 rpm. The cultures were then diluted 1/500 in 500 ml of selective LB and incubated for 12-16h at 37°C with shaking. The bacterial cells were harvested by centrifugation at 6,000xg for 20 min at 4°C and the plasmids were purified using the EndoFree™ Plasmid Mega Kit (Qiagen, Valencia, CA) according to the directions supplied by the manufacturer. Purified plasmid for DNA immunizations were resuspended in endotoxin-free saline (0.15M) and stored immediately at –20°C.

Expression of PLP was performed by transfection of the COS-7 cell line (CRL-1651) with linearized pEF1-PLP. The plasmid was linearized by digestion with the restriction enzyme *Pme*I (Invitrogen, San Diego, CA) for 16-18h at 37°C. Following digestion, the reaction was incubated at 65°C for 20 min to inactivate the enzyme. The linearized pEF1-PLP was purified using the QIAquick Spin Kit (Qiagen, Valencia, CA) according to the procedure provided with the kit. COS-7 cells were cultured in T-75 tissue culture flasks (CoStar, Corning, Inc., Corning, NY) in Dulbecco's Modification of Eagle's Medium (DMEM; Cellgro, Herndon, VA) containing 10% fetal bovine serum (JRH Biosciences, Lenexa, KS) at 37°C in 5% CO_2. A 12-well tissue culture plate was seeded with 4x10e4 COS-7 cells per well. When the cells reached 40-80% confluency (usually in 24h), transfections were performed using a range of DNA concentrations and various amounts of Effectene Transfection Reagent (Qiagen, Valencia, CA) according to the directions provided by the manufacturer. Twenty-four hours post-transfection, the medium was replaced with fresh DMEM containing 600 μg/ml of G418 (Invitrogen, San Diego, CA) for selection of transfected cells. Expression of PLP was confirmed by indirect immunofluorescence analysis using a rabbit anti-PLP polyclonal serum (Biogenesis, United Kingdom). Alternatively, an anti-*Myc* or anti-His antibody can also be used to test expression of the construct.

The efficacy of the pEF1-IL8 construct was confirmed by a transient transfection in COS-7 cells. IL-8 production was quantified in cell culture supernatants by a standard curve using IL-8 specific monoclonal antibodies and IL-8.

Example 26 Preparation of membrane vesicles for panning

Transfected cells are gently dissociated from the flask using Cell Dissociation Buffer (Life Technologies, Gaithersburg, MD), washed three times in sterile 0.01M phosphate buffered saline, pH 7.4 and maintained at 4°C during manipulations. The cell suspension is passed through a 30-gauge needle ten times then centrifuged at 2,000xg for 10 min. The supernatant containing membrane fragments is collected and sonicated on ice for 30s two times and pausing for 30s between each sonication. The sizes and size distribution of membrane vesicles are assessed using a particle sizer (Particle Sizing Systems, Holland, PA). Only preparations containing vesicles between 40-100nm are used for panning phage antibody libraries. For panning, phage antibodies that bind to right-side out membrane vesicles or non-sealed membranes containing glycoproteins are captured by binding to wheat germ agglutinin (Lindsay *et al.* (1981) *Biochim Biophys Acta*, 640:791-801) coated magnetic latex. The preparation of lectin magnetic latex is performed in a similar fashion as avidin magnetic latex described in Example 10.

Alternatively, a biotin-phospholipid, *N*-((6-biotinoyl)amino)hexanoyl)-1,2-dihexadecanoyl-*sn* -glycero-3-phosphoethanolamine, triethylammonium salt (biotin-X DHPE; Molecular Probes, Inc, Eugene, OR), is dissolved in chloroform/methanol (2:1) in a test tube then evaporated to dryness under nitrogen (Rivnay *et al.* (1987) *Methods in Enzymology* 149:119-123, Academic Press, Inc., San Diego, CA). The supernatant containing cell membranes is added to the tube coated with biotin-phospholipids and sonicated as described above. Phage antibodies binding to the biotinylated vesicles are captured using avidin magnetic latex as described in Example 13.

Example 27 ELISA Assays for Serum Titers and Specific Antibody Detection

In general, antigens were immobilized either directly by absorption onto microtiter plate wells or antigens were biotinylated and bound to microtiter plate wells that already had streptavidin or NeutrAvidin™ (Pierce, Rockford, IL) bound to them by adsorption. In the case of direct adsorption, fifty microliters of troponin complex antigen (Bio-tech International Inc., Seattle, WA, 2 μg/ml) were adsorbed to wells of a 96-well microwell plate (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ) overnight at 4°C. Excess antigen was removed and the wells were blocked with 200 μl of 10 mM TRIS, 150 mM NaCl, 10 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 0.1% polyvinyl alcohol, 1% bovine serum albumin, 0.1% NaN_3, pH 8.0 (block buffer). After washing the plates three times with 300 μl of BBST (20 mM borate, 150 mM NaCl, 0.1% NaN_3, 0.02% Tween 20), the wells were filled with 100 μl of 2-fold serial dilutions (in block buffer) of the mouse sera beginning at a 1:50 dilution. The wells were incubated with sera for 1h at ambient temperature then washed as described above. After washing, the wells were filled with 100 μl of alkaline phosphatase-conjugated anti-human IgG Fc specific antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) diluted 1:250 in block buffer. After 1h of incubation at ambient temperature, the wells were washed and 200 μl of the substrate phenolphthalein monophosphate (JBL Scientific Inc., San Luis Obispo, CA), 6 mg/ml in 0.2 M 2-amino-2-methyl-1-propanol, 0.5 M Tris, pH 10.2) was added to each well. The kinetics was measured for 1 min at an optical density of 560 nm (Molecular Devices, Sunnyvale, CA). The titer of the serum was the reciprocal of the dilution at which the signal was greater than two times that of the background signal (Table 7). Test bleeds (TB) were drawn nine days after three biweekly immunizations with TIC antigen (TB-1), nine days after the 4^{th} immunization (TB-2), and nine days after the 5^{th} immunization (TB-3).

Table 7 Antibody titers

Mouse ID	TB-1	TB-2	TB-3
A	-	-	-
B	-	3,200	12,800
C	-	-	-
D	-	50	200
E	-	-	-

To detect specific antibodies that had been expressed in E. coli and either obtained directly from disrupted cells or as purified antibody, biotinylated antigens troponin TIC complex, oxidized troponin I and IL-8 (Examples 9 and 33) were immobilized on 96- well polystyrene microtiter plates that had streptavidin or NeutrAvidin™ immobilized on them using a concentration of 10^{-8} M biotinylated antigen. Dilutions of the antibody preparations were incubated for one hour with the immobilized antigens and unbound antibody was washed away as described above. The presence of bound antibody was detected using goat-anti-human antibody conjugated to alkaline phosphatase that is specific for the human kappa chain (Southern Biotechnology Associates, Birmingham, AL) and the method described above.

Example 28 Isolation of RNA from bone marrow from mice immunized with oxidized troponin I

Five HCo7 mice were immunized with oxidized troponin I (Example 32) as described in Example 1. Immediately post-splenectomy, muscle and connective tissues were removed from the femur. The cleaned femur was transferred to a sterile 35mm petri dish containing 0.6 ml of sample lysis buffer (Buffer RLT; QIAamp RNA Isolation Kit, Qiagen, Valencia, CA). The bone was split longitudinally with a sterile No. 15 scalpel and the exposed marrow was teased out of the bone into the lysis buffer using a 27 gauge needle. The head of the femur was further split and scraped to remove all remaining marrow. The lysis buffer containing bone marrow cells and small bone fragments was transferred to a QIAshredder spin column for complete homogenization of cells and processed according to the manufacturer's protocol. Bone fragments were effectively removed from the sample as they failed to enter the QIAshredder spin column. Subsequent isolation of total RNA using the QIAamp spin

column and a DNase1 digestion step were performed according to the procedures provided by the manufacturer.

Example 29 DNA immunizations

Plasmid DNA (pEF1-IL8, Example 25 and pEF1-PLP, Example 25) were suspended in endotoxin-free saline at a concentration of 1 mg/ml for DNA immunizations. Prior to injections, the mice were anaesthetized and the hind limbs were shaved to allow improved accessibility to the tibialis anterior muscle. Fifty microliters of the DNA suspension was injected slowly into each muscle (50 μg of DNA per injection site) using a 27 gauge needle. Mice were immunized on day 0 and day 14 and test bleeds were performed on day 14 (TB-1) and day 21 (TB-2). RNA was isolated on day 21 by the procedure described in Example 1.

Serum titer assays were performed on pre-immune sera collected prior to immunizations and test bleeds as described in Example 27 for the mice immunized with pEF1-IL8 The plates were coated with unlabeled IL8, and sera were tested at a starting dilution of 1:20.

Table 8 Antibody Titers

Mouse ID	Pre-immune	TB-1	TB-2
A	-	-	20
B	-	-	-
C	-	-	-

Example 30. Amplification of human antibody sequence cDNA by PCR from spleens of mice exhibiting no serum titer to TIC

Five HCo7 mice were immunized with troponin complex (Bio-tech International Inc., Seattle, WA) as described in Example 1. The RNA from spleens C and E described in Example 27 was purified as described in Example 1 and cDNA was made as described in Example 2. The cDNA was amplified by PCR as described in Example 3 for HCo7 mice except 5 μL of cDNA was used for the double stranded PCR of the heavy chain for spleen E, and the 3' primer for the double stranded PCR was done using oligonucleotide 1008 for both spleens.

Oligonucleotide 1008 5'-CAC CGT CAC CGG TTC GGG GA

Example 31. Amplification of human antibody RNA by one step reverse transcription-polymerase chain reaction (RT-PCR)

The total RNA purified from spleen (Example 29 for IL8) or bone marrow (Example 28) described above was used directly as template. The RNA from the bone marrow of mice A and E were pooled and RNA from the bone marrow of mice C and D were pooled for the bone marrow PCR. Superscript™ One -Step RT-PCR with PLATINUM® Taq (Gibco/BRL, Gaithersburg, MD) was used as described in the product insert. The oligonucleotides described for HCo7 mice in Example 3 were used except oligonucleotide 1008 (Example 30) was the 3' oligonucleotide for the heavy chain instead of oligonucleotide 952. One 25μL reaction was performed for each primer pair with 12.5 pmol of 5' primer, 12.5 pmol of 3' primer, and 0.625μg of RNA. Amplification was done using a GeneAmp® 9600 thermal cycler (Perkin Elmer, Foster City, CA) with the following program: 45°C for 30 min; 94 °C for 2 min; 40 cycles of 94 °C for 15 sec, 55 °C for 30 sec, and 72 °C for 60sec; 72 °C for 7 min; 4 °C. The dsDNA products of the PCR process were then subjected to asymmetric PCR using only 3' primer to generate substantially only the anti-sense strand of the target genes, as described in Example 3.

Example 32. Preparation of oxidized troponin I

Cardiac troponin I (Bio-tech International Inc., Seattle, WA) was dialyzed extensively against 100mM potassium phosphate, 50mM potassium borate, 1M NaCl, pH 7.0. After dialysis, 1M H_2O_2 was added to the protein at a final concentration of

20mM, and the mixture was incubated at room temperature for 30 minutes. The troponin I oxidized solution was transferred to dialysis tubing and dialyzed against 100mM potassium phosphate, 50mM potassium borate, 1M NaCl, 1.4ug/ml Catalase, pH 7.0 for 3hr at room temperature. After 3hr, the protein was dialyzed twice against 100mM potassium phosphate, 50mM potassium borate, 1M NaCl, pH 7.0, then once against 100mM potassium phosphate, 50mM potassium borate, 0.5M NaCl, pH 7.0 for at least 4 hr each at 2-8°C.

Example 33. Preparation of biotinylated troponin complex (TIC) and biotinylated oxidized troponin

Troponin complex and oxidized troponin were dialyzed against a minimum of 100 volumes of 20 mM borate, 150 mM NaCl, pH 8 (BBS) with 2mM CaCl2 at 2-8 °C for at least 4 hr. The buffer was changed at least once prior to biotinylation. Troponin complex and oxidized troponin were reacted with biotin-XX-NHS ester (Molecular Probes, Eugene, OR, stock solution at 40 mM in dimethylformamide) at a final concentration of 0.1 mM for TIC and 0.2mM for oxidized troponin for 1 hr at room temperature. After 1 hr, the proteins were extensively dialyzed into BBS with 2mM $CaCl_2$ to remove unreacted small molecules.

Example 34. Enrichment of polyclonal phage from mice immunized with TIC but exhibiting no serum titers of antibody

The RNA from spleens C and E (Example 27) was amplified by PCR (Example 30), and first round antibody phage was prepared as described in Example 7 using BS47 uracil template. The mice from where the spleens originated did not have an antibody serum titer in the test bleeds. Four electroporations of mutagenesis DNA were done from 2 different spleens (2 electroporations from each spleen) yielding 4 different phage samples. Phage were set up for the first round of panning by mixing 0.92mL phage, 30µL 300mg/mL BSA, 2µL 1M $CaCl_2$, 50µL 1M TRIS, pH 8.0 and 10 µL 10^{-7}M TIC-biotin (Example 33), and incubating overnight at 2-8°C. The antibody phage samples were panned with avidin magnetic latex as described in Example 13. The only difference is the panning buffer also contained 2mM $CaCl_2$. This panning buffer was used for every panning step described in this example.

The resulting 2nd round antibody phage samples were enriched for polyvalent display by panning with 7F11 magnetic latex as described in Example 13. Panning with TIC-biotin was set up for each sample by mixing 900 µL 7F11/decapeptide

enriched phage, 2μL 1M $CaCl_2$, 100 μL panning buffer, and 10 μL 10^{-7} M TIC-biotin. After overnight incubation at 2-8°C, the phage samples were panned with avidin magnetic latex as described above.

The resulting 3rd round antibody phage samples were again enriched for polyvalent display and the eluted phage were set up with TIC-biotin as described above. After overnight incubation at 2-8°C, the phage samples were panned with avidin magnetic latex as described above. Aliquots of each sample were plated on 100mm LB agar plates to determine the percentage of kappa positives (Example 12). The percentage of kappa positives for the 3rd round of panning was between 91-97%.

The 4th round antibody phage samples were titered as described in Example 13. The two phage samples from each spleen were pooled to give spleen C pool and spleen E pool. The pooled antibody phage was set up in duplicate for a 4th round of functional panning as described above using 900 μL panning buffer, 100 μL 4th round pooled-antibody phage. One sample (foreground) received 10 μL 10^{-7}M TIC-biotin and the other sample (background) did not receive TIC-biotin and served as a blank to monitor non-specific binding of phage to the magnetic latex. After overnight incubation at 2-8°C, the phage samples were panned with avidin magnetic latex as described above. The next day, the 5th round antibody phage was eluted and the number of plaques was counted on the foreground and background plates. The foreground:background ratio was 148:1 for spleen C and 190:1 for spleen E. The antibody phage populations were subcloned into the expression vector and electroporated as described in Example 15, except oligonucleotides 1161 and 1178 were used to amplify the antibody gene insert of spleen C and oligonucleotides 1161 and 1182 were used to amplify the antibody gene insert of spleen E

Primer 1161 5'-TC GCT GCC CAA CCA GCC ATG GCC

Primer 1178 5'-GT GAT AAA CTA CCG CAT TA AAG CTT ATC GAT GAT AAG CTG TCA A TTA GTG ATG GTG ATG GTG ATG AGA TTT GG

Primer 1182 5'- GT GAT AAA CTA CCG CAT TA AAG CTT ATC GAT GAT AAG CTG TCA A TTA GTG ATG GTG ATG GTG ATG ACA TTT GG

Fig. 8 shows ELISA data of six monoclonals from the above experiment confirming specific binding to the target antigen.

Example 35 Enrichment of polyclonal phage from mice immunized with DNA

RNA purified from the spleens of 3 HCo7 mice immunized with the pEF1-IL8 plasmid (Example 29) was amplified by PCR (Example 31), and first round antibody phage was prepared as described in Example 7 using BS47 uracil template. Six electroporations of mutagenesis DNA were done (2 electroporations from each spleen) yielding 6 different phage samples. Phage were set up for the first round of panning by mixing 0.92mL phage, 52.5μL 5% casein, 30μL 300mg/mL BSA, 50μL 1M TRIS, pH 8.0 and 10 μL 10^{-7}M IL8-biotin (Example 9) and incubating 3hr at room temperature. The antibody phage samples were panned with avidin magnetic latex as described in Example 13. The only difference is the panning buffer also contained 0.25% casein (Sigma, St. Louis, MO). This panning buffer was used for every panning step described in this example.

The resulting 2nd round antibody phage samples were enriched for polyvalent display by panning with 7F11 magnetic latex as described in Example 13. Panning with IL8-biotin was set up for each sample by mixing 900 μL 7F11/decapeptide enriched phage, 100 μL panning buffer, and 10 μL 10^{-7} M IL8-biotin. After 3hr incubation at room temperature, the phage samples were panned with avidin magnetic latex as described above.

The resulting 3rd round antibody phage samples were again enriched for polyvalent display and the eluted phage were set up with IL8-biotin as described above. After 3hr incubation at room temperature, the phage samples were panned with avidin magnetic latex as described above. Aliquots of each sample were plated on 100mm LB agar plates to determine the percentage of kappa positives (Example 12). The percentage of kappa positives for the 3rd round of panning was between 93-100%.

The 4th round antibody phage samples were titered as described in Example 13 and the phage were mixed into one pool. The pooled antibody phage was set up in duplicate for a 4th round of functional panning as described above using 900 μL panning buffer, 100 μL 4th round pooled-antibody phage. One sample (foreground) received 10 μL 10^{-7}M IL8-biotin and the other sample (background) did not receive IL8-biotin and served as a blank to monitor non-specific binding of phage to the magnetic latex. After overnight incubation at 2-8°C, the phage samples were panned with avidin magnetic latex as described above. The next day, the 5th round

antibody phage was eluted and the number of plaques was counted on the foreground and background plates. The foreground:background ratio was 109:1. The antibody phage populations were subcloned into the expression vector and electroporated as described in Example 15, except oligonucleotides 1161 and 1178 were used to amplify the antibody gene inserts (Example 34).

A randomly selected IL-8 monoclonal antibody (53B10) and the IL-8 polyclonal antibody (53B omni) were expressed and purified as described in Example 16. The solution phase equilibrium dissociation constants were determined for both preparations using a KinExA™ 3000 instrument (Sapidyne Instruments Inc., Boise, ID) following the protocols and parameters suggested by the manufacturer. Briefly, an antibody solution is allowed to come to equilibrium with serial dilutions of native antigen (IL-8) (see Fig. 9). Free antibody is then captured and quantified on a solid phase support that is coated with biotinylated antigen (Example 9). Using the antigen and free antibody concentrations, data analysis software designed by Sapidyne calculates the K_d. Assays of the polyclonal preparation derived from the DNA-immunized mice resulted in a K_d determination of 2.8 pM (range 138pM - 9.1fM). The selected monoclonal has a K_d of 2.9pM (range 379pM – 9fM). For comparison, a monoclonal from a mouse immunized with purified IL-8 (MED002 1.25.2) has a K_d of 16pM (range 85pM – 55fM).

Example 36 Enrichment of polyclonal phage from mice immunized with oxidized troponin with the RNA purified from bone marrow

RNA purified from the bone marrow of four HCo7 mice was amplified by PCR (Example 31), and first round antibody phage was prepared as described in Example 7 using BS47 uracil template. Four electroporations of mutagenesis DNA were done (2 electroporations from each spleen combination) yielding 4 different phage samples. Phage were set up for the first round of panning by mixing 0.92mL phage, 52.5µL 5% casein, 30µL 300mg/mL BSA, 50µL 1M TRIS, pH 8.0 and 10 µL 10^{-7}M oxidized troponin I-biotin (Example 33, ox-TnI-biotin) and incubating 3hr at room temperature. The antibody phage samples were panned with avidin magnetic latex as described in Example 13. The only difference is high salt conjugate diluent (1% bovine serum albumin (from 30% BSA, Bayer, Kankakee, IL), 10 mM MOPS, 650 mM NaCl, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 0.25% casein, 0.1% polyvinyl alcohol (80% hydrolyzed, Aldrich Chemical Co., Milwaukee, WI), pH 7.0).was used instead

of the panning buffer. This buffer (HSCD) was used for every avidin magnetic latex panning step described in this example. The panning buffer described in Example 35 was used for the 7F11 enrichment panning.

The resulting 2nd round antibody phage samples were enriched for polyvalent display by panning with 7F11 magnetic latex as described in Example 13. Panning with ox-TnI-biotin was set up for each sample by mixing 900 μL 7F11/decapeptide enriched phage, 100 μL panning buffer, and 10 μL 10^{-7} M ox-TnI-biotin. After 3hr incubation at room temperature, the phage samples were panned with avidin magnetic latex as described above.

The resulting 3rd round antibody phage samples were again enriched for polyvalent display and the eluted phage were set up with ox-TnI-biotin as described above. After 3hr incubation at room temperature, the phage samples were panned with avidin magnetic latex as described above. Aliquots of each sample were plated on 100mm LB agar plates to determine the percentage of kappa positives (Example 12). The percentage of kappa positives for the 3rd round of panning was between 98-100%.

The 4th round antibody phage samples were titered as described in Example 13 and the phage were mixed into one pool. The pooled antibody phage was set up in duplicate for a 4th round of functional panning as described above using 900 μL panning buffer, 100 μL 4th round pooled-antibody phage. One sample (foreground) received 10 μL 10^{-7}M ox-TnI-biotin and the other sample (background) did not receive ox-TnI-biotin and served as a blank to monitor non-specific binding of phage to the magnetic latex. After overnight incubation at 2-8°C, the phage samples were panned with avidin magnetic latex as described above. The next day, the 5th round antibody phage was eluted and the number of plaques was counted on the foreground and background plates. The foreground:background ratio was 2.5:1.

The 5th round antibody phage was set up in duplicate for a 5th round of functional panning as described above using 900 μL panning buffer, 100 μL 5th round antibody phage. One sample (foreground) received 10 μL 10^{-7}M ox-TnI-biotin and the other sample (background) did not receive ox-TnI-biotin and served as a blank to monitor non-specific binding of phage to the magnetic latex. After 3hr incubation at room temperature, the phage samples were panned with avidin magnetic latex as described above. The next day, the 6th round antibody phage was eluted and

the number of plaques was counted on the foreground and background plates. The foreground:background ratio was 36:1. The antibody phage populations were subcloned into the expression vector and electroporated as described in Example 15, except oligonucleotides 1161 and 1178 were used to amplify the antibody gene inserts (Example 34). ELISA data of a crude population of cells expressing polyclonal antibody is shown in Fig. 10 confirming specific binding.

Example 37 DNA sequence analysis of random clones.

Six random clones, each, were picked from the spleenC and spleenE subcloned populations (Example 34) and used to inoculate 50ml cultures for plasmid isolation and subsequent DNA sequencing of the antibody inserts. After overnight growth in 2xYT (10μg/ml tetracycline) at 37°C, the recombinant plasmid was purified using a Qiagen Plasmid Midi kit (Qiagen, Valencia, CA) following manufacturer's recommendations. The sequence corresponding to the kappa and heavy chain variable and constant regions for each monoclonal was determined at MacConnell Research (San Diego, CA). Sequencing was done by the dideoxy chain termination method using a Sequatherm sequencing kit (Epicenter Technologies, Madison, WI), oligonucleotide primers C and D (Table 3) that bind on the 5' and 3' side of the Fab cassette in the pBR vector, respectively, and a LI-COR 4000L automated sequencer (LI-COR, Lincoln, NE).

TRO005 KAPPA CHAINS DNA SEQUENCES

1C.B1

CGAAATAGTGATGACGCAgtcTCCAGCCACCCTGTCTTTGTCTCCAGGGGAAAGAGCCACCCTCTCCTG
CAGGGCCAGTCAGAGTGTTTACAGCTACTTAGTCTGGTACCAACAGAAACCTGGCCAGGCTCCCAGGCT
CCTCATCTATGATGCATCCAACAGGGCCACTGGCATCCCAGCCAGGTTCAGTGGCAGTGGGTCTGGGAC
AGACTTCACTCTCACCATCAGCAGCCTAGAGCCTGAAGATTTTGCATTTTATTACTGTCAGCAGCGTAc
GAACCGGCCGTACACTTTTGGCCAGGGGACCAAGCTGGAGATCAAACGAACTGTGGCTGCACCATCTGT
CTTCATCTTCCCGCCATCTGATGAGCAGTTGAAATCTGGAACTGCCTCTGTTGTGTGCCTGCTGAATAA
CTTCTATCCCAGAGAGGCCAAAGTACAGTGGAAGGTGGATAACGCCCTCCAATCGGGTAACTCCCAGGA
GAGTGTCACAGAGCAGGACAGCAAGGACAGCACCTACAGCCTCAGCAGCACCCTGACGCTGAGCAAAGC
AGACTACGAGAAACACAAAGTCTACGCCTGCGAAGTCACCCATCAGGGCCTGAGCTCGCCCGTCACAAA
GAGCTTCAACAGGGGAGAGTCTTATCCATATGATGTGCCAGATTATGCGAGC

1C.C2

GAGCTCGTGATGACACAGTCTCCAGCCACCCTGTCTTTGTCTCCAGGGGAAAGAGCCACCCTCTCCTGC
AGGGCCAGTCAGAGTGTTTACAGCTACTTAGTCTGGTACCAACAGAAACCTGGCCAGGCTCCCAGGCTC
CTCATCTATGATGCATCCAACAGGGCCACTGGCATCCCAGCCAGGTTCAGTGGCAGTGGGTCTGGGACA
GACTTCACTCTCACCATCAGCAGCCTAGAGCCTGAAGATTTTGCAGTTTATTACTGTCAGCAGCGTAcG

AACTGGCCGTGGACGTTCGGCCAAGGGACCAAGGTGGAAATCAAACGAACTGTGGCTGCACCATCTGTC
TTCATCTTCCCGCCATCTGATGAGCAGTTGAAATCTGGAACTGCCTCTGTTGTGTGCCTGCTGAATAAC
TTCTATCCCAGAGAGGCCAAAGTACAGTGGAAGGTGGATAACGCCCTCCAATCGGGTAACTCCCAGGAG
AGTGTCACAGAGCAGGACAGCAAGGACAGCACCTACAGCCTCAGCAGCACCCTGACGCTGAGCAAAGCA
GACTACGAGAAACACAAAGTCTACGCCTGCGAAGTCACCCATCAGGGCCTGAGCTCGCCCGTCACAAAG
AGCTTCAACAGGGGAGAGTCTTATCCATATGGTGTGCCAGATTATGCGAGC

1C.C6

GAGCTCGTGATGACCCAGACTCCACTCTCCCTGTCTTTGTCTCCAGGGGAAAGAGCCACCCTCTCCTGC
AGGGCCAGTCAGAGTATTTACAACTACTTAGCCTGGTACCAACAGAAACCTGGCCAGGCTCCCAGGCTC
CTCATCTATGATGCATCCAACAGGGCCACTGGCATCCCAGCCAGGTTCAGTGGCAGTGGGTCTGGGACA
GACTTCACTCTCACCATCAGCAGCCTAGAGCCTGAAGATTTTGCAGTTTATTACTGTCAGCAGCGTAcG
AACTGGCCGTGGACGTTCGGCCAAGGGACCAAGGTGGAAATCAAACGAACCGTGGCTGCACCATCTGTC
TTCATCTTCCCGCCATCTGATGAGCAGTTGAAATCTGGAACTGCCTCTGTTGTGTGCCTGCTGAATAAC
TTCTATCCCAGAGAGGCCAAAGTACAGTGGAAGGTGGATAACGCCCTCCAATCGGGTAACTCCCAGGAG
AGTGTCACAGAGCAGGACAGCAAGGACAGCACCTACAGCCTCAGCAGCACCCTGACGCTGAGCAAAGCA
GACTACGAGAAACACAAAGTCTACGCCTGCGAAGTCACCCATCAGGGCCTGAGCTCGCCCGTCACAAAG
AGCTTCAACAGGGGAGAGTCTTATCCATATGATGTGCCAGATTATGCGAGC

1C.C8

GAAATTGTGTTGACGCAgtcTCCAGGCACCCTGTCTTTGTCTCCAGGGGAAAGAGCCACCCTCTCCTGC
AGGGCCAGTCAGAGTATTTACAACTACTTAGCCTGGTACCAACAGAAACCTGGCCAGGCTCCCAGGCTC
CTCATCTATGATGCATCCAACAGGGCCACTGGCATCCCAGCCAGGTTCAGTGGCAGTGGGTCTGGGACA
GACTTCACTCTCACCATCAGCAGCCTAGAGCCTGAAGATTTTGCAGTTTATTACTGTCAGCAGCGTAcG
AACTGGCCGTGGACGTTCGGCCAAGGGACCAAGGTGGAAATCAAACGAACTGTGGCTGCACCATCTGTC
TTCATCTTCCCGCCATCTGATGAGCAGTTGAAATCTGGAACTGCCTCTGTTGTGTGCCTGCTGAATAAC
TTCTATCCCAGAGAGGCCAAAGTACAGTGGAAGGTGGATAACGCCCTCCAATCGGGTAACTCCCAGGAG
AGTGTCACAGAGCAGGACAGCAAGGACAGCACCTACAGCCTCAGCAGCACCCTGACGCTGAGCAAAGCA
GACTACGAGAAACACAAAGTCTACGCCTGCGAAGTCACCCATCAGGGCCTGAGCTCGCCCGTCACAAAG
AGCTTCAACAGGGGAGAGTCTTATCCATATGATGTGCCAGATTATGCGAGC

1C.D7

GAGCTCGTGATGACCCAGTCTCCAGCCACCCTGTCTTTGTCTCCAGGGGAAAGAGCCACCCTCTCCTGC
AGGGCCAGTCAGAGTATTTACAACTACTTAGCCTGGTACCAACAGAAACCTGGCCAGGCTCCCAGGCTC
CTCATCTATGATGCATCCAACAGGGCCACTGGCATCCCAGCCAGGTTCAGTGGCAGTGGGTCTGGGACA
GACTTCACTCTCACCATCAGCAGCCTAGAGCCTGAAGATTTTGCAGTTTATTACTGTCAGCAGCGTAcG
AACTGGCCGTGGACGTTCGGCCAAGGGACCAAGGTGGAAATCAAACGAACTGTGGCTGCACCATCTGTC
TTCATCTTCCCGCCATCTGATGAGCAGTTGAAATCTGGAACTGCCTCTGTTGTGTGCCTGCTGAATAAC
TTCTATCCCAGAGAGGCCAAAGTACAGTGGAAGGTGGATAACGCCCTCCAATCGGGTAACTCCCAGGAG
AGTGTCACAGAGCAGGACAGCAAGGACAGCACCTACAGCCTCAGCAGCACCCTGACGCTGAGCAAAGCA
GACTACGAGAAACACAAAGTCTACGCCTGCGAAGTCACCCATCAGGGCCTGAGCTCGCCCGTCACAAAG
AGCTTCAACAGGGGAGAGTCTTATCCATATGATGTGCCAGATTATGCGAGC

1C.E8

GAGCTCGTGATGACCCAGACTCCACTCTCCCTGTCTTTGTCTCCAGGGGAAAGAGCCACCCTCTCCTGC
AGGGCCAGTCAGAATGTTTACAGCTACTTAGCCTGGTACCAACAGAAACCTGGCCAGGCTCCCAGGCTC
CTCATCTATGATGCATCCAACAGGGCCCCTGGCATCCCAGCCAGGTTCAGTGGCAGTGGGTCTGGGACA
GACTTCACTCTCACCATCAGCAGCCTAGAGCCTGAAGATTTTGCAGTTTATTACTGTCAGCAGCGTAcG
AACTGGCCGTGGACGTTCGGCCAAGGGACCAAGGTGGAAATCAAACGAACTGTGGCTGCACCATCTGTC
TTCATCTTCCCGCCATCTGATGAGCAGTTGAAATCTGGAACTGCCTCTGTTGTGTGCCTGCTGAATAAC
TTCTATCCCAGAGAGGCCAAAGTACAGTGGAAGGTGGATAACGCCCTCCAATCGGGTAACTCCCAGGAG
AGTGTCACAGAGCAGGACAGCAAGGACAGCACCTACAGCCTCAGCAGCACCCTGACGCTGAGCAAAGCA
GACTACGAGAAACACAAAGTCTACGCCTGCGAAGTCACCCATCAGGGCCTGAGCTCGCCCGTCACAAAG
AGCTTCAACAGGGGAGAGTCTTATCCATATGATGTGCCAGATTATGCGAGC

3E.1

GAAATAGTGATGACGCAgTcTCCAGGCACCCTGTCTTTGTCTCCAGGGGAAAGAGCCACCCTCTCCTGC
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3E.2

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CAACAGTATAATAGTTACCCATTCACTTTCGGCCCTGGGACCAAAGTGGATATCAAACGAACTGTGGCT
GCACCATCTGTCTTCATCTTCCCGCCATCTGATGAGCAGTTGAAATCTGGAACTGCCTCTGTTGTGTGC
CTGCTGAATAACTTCTATCCCAGAGAGGCCAAAGTACAGTGGAAGGTGGATAACGCCCTCCAATCGGGT
AACTCCCAGGAGAGTGTCACAGAGCAGGACAGCAAGGACAGCACCTACAGCCTCAGCAGCACCCTGACG
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3E.3

GACATCCAGATGATCCAGTCTCCATCCTCCCCGTCTGCATCTGTAGGAGACAGAGTCACCATCACTTGC
CGGGCAAGTCAGGGCATTAGCAGTGCTTTAGCCTGGTATCAGCAGAAACCAGGGAAAGCTCCTAAGCTC
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3E.4

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3E.8

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3E.9

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TRO005 HEAVY CHAINS DNA SEQUENCES

1C.B1

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1C.C2

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1C.C6

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1C.C8

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1C.D7

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1C.E8

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3E.1

CAGGTGCAGCTGGTGCAGTCTGGGGGAGGCGTGGTCCAGTCTGGGAGGTCCCTGAGACTCTCCTGT
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CACCATCACCATCAC

3E.2

CAGGTGCAGCTGGTGCAGTCTGGGGCAGAGGTGAAAAAGCCCGGGGAGTCTCTGAAGATCTCCTGT
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3E.3

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CACCATCACCATCAC

3E.4

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CACCATCACCATCAC

3E.8

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CACCATCACCATCAC

3E.9

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CACCATCACCATCAC

Alignment: TRO005 HuMab Kappa Chain

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          1                                                  50
1CB1K     EIVMTQSPAT LSLSPGERAT LSCRASQSVY S.YLVWYQQK PGQAPRLLIY
1CC2K     ELVMTQSPAT LSLSPGERAT LSCRASQSVY S.YLVWYQQK PGQAPRLLIY
3E1K      EIVMTQSPGT LSLSPGERAT LSCRASQSVS SRYLAWYQQK PGQAPRLLIY
1CC8K     EIVLTQSPGT LSLSPGERAT LSCRASQSIY N.YLAWYQQK PGQAPRLLIY
1CD7K     ELVMTQSPAT LSLSPGERAT LSCRASQSIY N.YLAWYQQK PGQAPRLLIY
1CE8K     ELVMTQTPLS LSLSPGERAT LSCRASQNVY S.YLAWYQQK PGQAPRLLIY
1CC6K     ELVMTQTPLS LSLSPGERAT LSCRASQSIY N.YLAWYQQK PGQAPRLLIY
3E2K      NIQMTQSPSS LSASVGDRVT ITCRASQGIS S.WLAWYQQK PEKAPKSLIY
3E3K      DIQMIQSPSS PSASVGDRVT ITCRASQGIS S.ALAWYQQK PGKAPKLLIY
3E4K      EIVMTQSPGT LSLSPGERAT LSCRASQSVS SRYLAWYQQK PGQAPRLLIY
3E8K      AIQLTQSPSS LSASVGDRVT ITCRASQGIS S.ALAWYQQK PEKAPKLLIY
3E9K      ELVMTQSPSS LSASVGDRVT ITCRASQGIS S.WLAWYQQK PEKAPKSLIY

          51                                                 100
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1CC2K     DASNRATGIP ARFSGSGSGT DFTLTISSLE PEDFAVYYCQ QRTNWPWTFG
3E1K      GASSRATGIP DRFSGSGSGT DFTLAISRLE PEDFAVYFCQ QYG.SSITFG
1CC8K     DASNRATGIP ARFSGSGSGT DFTLTISSLE PEDFAVYYCQ QRTNWPWTFG
1CD7K     DASNRATGIP ARFSGSGSGT DFTLTISSLE PEDFAVYYCQ QRTNWPWTFG
1CE8K     DASNRAPGIP ARFSGSGSGT DFTLTISSLE PEDFAVYYCQ QRTNWPWTFG
1CC6K     DASNRATGIP ARFSGSGSGT DFTLTISSLE PEDFAVYYCQ QRTNWPWTFG
3E2K      AASSLQSGVP SRFSGSGSGT DFTLTISSLQ PEDFATYYCQ QYNSYPFTFG
3E3K      DASSLESGVP SRFSGSGSGT DFTLTISSLQ PEDFATYYCQ QYNSYPLTFG
3E4K      GASSRATGIP DRFSGSGSGT DFTLAISRLE PEDFAVYFCQ QYG.SSITFG
3E8K      DASSLESGVP SRFSGSGSGT DFTLTISSLQ PEDFATYYCQ QYNSYPWTFG
3E9K      AASSLQSGVP SRFSGSGSGT DFTLTISSLQ PEDFATYYCQ QYNSYPITFG

          101                                                150
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1CC2K     QGTKVEIKRT VAAPSVFIFP PSDEQLKSGT ASVVCLLNNF YPREAKVQWK
3E1K      QGTRLEIKRT VAAPSVFIFP PSDEQLKSGT ASVVCLLNNF YPREAKVQWK
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3E8K      QGTKVEIKRT VAAPSVFIFP PSDEQLKSGT ASVVCLLNNF YPREAKVQWK
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          151                                                200
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1CC2K     VDNALQSGNS QESVTEQDSK DSTYSLSSTL TLSKADYEKH KVYACEVTHQ
3E1K      VDNALQSGNS QESVTEQDSK DSTYSLSSTL TLSKADYEKH KVYACEVTHQ
1CC8K     VDNALQSGNS QESVTEQDSK DSTYSLSSTL TLSKADYEKH KVYACEVTHQ
1CD7K     VDNALQSGNS QESVTEQDSK DSTYSLSSTL TLSKADYEKH KVYACEVTHQ
1CE8K     VDNALQSGNS QESVTEQDSK DSTYSLSSTL TLSKADYEKH KVYACEVTHQ
1CC6K     VDNALQSGNS QESVTEQDSK DSTYSLSSTL TLSKADYEKH KVYACEVTHQ
3E2K      VDNALQSGNS QESVTEQDSK DSTYSLSSTL TLSKADYEKH KVYACEVTHQ
3E3K      VDNALQSGNS QESVTEQDSK DSTYSLSSTL TLSKADYEKH KVYACEVTHQ
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          3E4K  VDNALQSGNS QESVTEQDSK DSTYSLSSTL TLSKADYEKH KVYACEVTHQ
          3E8K  VDNALQSGNS QESVTEQDSK DSTYSLSSTL TLSKADYEKH KVYACEVTHQ
          3E9K  VDNALQSGNS QESVTEQDSK DSTYSLSSTL TLSKADYEKH KVYACEVTHQ

                201                        225
        1CB1K  GLSSPVTKSF NRGESYPYDV PDYAS
        1CC2K  GLSSPVTKSF NRGESYPYGV PDYAS
         3E1K  GLSSPVTKSF NRGESYPYDV PDYAS
        1CC8K  GLSSPVTKSF NRGESYPYDV PDYAS
        1CD7K  GLSSPVTKSF NRGESYPYDV PDYAS
        1CE8K  GLSSPVTKSF NRGESYPYDV PDYAS
        1CC6K  GLSSPVTKSF NRGESYPYDV PDYAS
         3E2K  GLSSPVTKSF NRGESYPYDV PDYAS
         3E3K  GLSSPVTKSF NRGESYPYDV PDYAS
         3E4K  GLSSPVTKSF NRGESYPYDV PDYAS
         3E8K  GLSSPVTKSF NRGESYPYDV PDYAS
         3E9K  GLSSPVTKSF NRGESYPYDV PDYAS
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Alignment: TRO005 HuMab Heavy Chain

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                1                                                   50
        1CB1H  QVQLVESGGG VVQPGRSLRL SCAASGFTLR SYAMHWVRQA PGKGLEWVAV
        1CC2H  EVQLVQSGGG VVQPGRSLRL SCAASEFTFS NYAFHWVRQA PGKGLEWVAI
         3E1H  QVQLVQSGGG VVQSGRSLRL SCAASGITVR NYAMHWVRQV PGKGLEWVAV
        1CC8H  QVQLVQSGGG VVQPGRSLRL SCAASGFTFS NYAFHWVRQA PGKGLEWVAI
        1CD7H  QVQLVESGGG VVQPGRSLRL SCAASGFTFS NYAMHWVRQA PGKGLEWVAI
        1CE8H  QVQLVQSGGG VVQPGRSLRL SCAASGFTFS NYAFHWVRQA PGKGLEWVAI
        1CC6H  EVQLVQSGGG LVQPGGSLRL SCAASGFTFS NYAMSWVRQA PGKGLEWVSA
         3E2H  QVQLVQSGAE VKKPGESLKI SCKGSGYSFT NYWIGWVRQM PGKGLEWMGF
         3E3H  QVQLVQSGAE VKKPGESLKI SCKGSGYSFT NYWIGWVRQM PGKGLEWMGF
         3E4H  QVQLVQSGGG VVQSGRSLRL SCAASGITVR NYAMHWVRQV PGKGLEWVAV
         3E8H  QVQLVESGGG VVQPGRSLRL SCAASGFTFR RYGMHWVRQA PGKGLEWVAV
         3E9H  QVQLVQSGAE VKKPGESLKI SCKGSGYSFT NYWIGWVRQM PGKGLEWMGI

                51                                                  100
        1CB1H  ISYDGSYKSY ADSVKGRFIS SRDNSKNTLS LQMNSLRAED TAVYFCARAM
        1CC2H  ISYDGSHKYY ADSVTGRFTI SRDNSKNTLY LQMNSLRAED TAVYYCARAM
         3E1H  ISYDGSNKYY ADSVKGRFTL SRDNSKNTLY LQMNSLRAED TAVYYCARED
        1CC8H  ISYDGSHKYY ADSVTGRFTI SRDNSKNTLY LQMNSLRAED TAVYYCARAM
        1CD7H  ISYDGTYKYY ADSVKGRFTI SRDNSKNTLY LQMNSLRAED TAVYYCARAM
        1CE8H  ISYDGSHKYY ADSVTGRFTT SRDNSKNTLY LQMNSLRAED TAVYYCARAM
        1CC6H  INYGGGSTYY ADSVKGRFTI SRDNSKNTLY LQMNSLRAED TAVYYCAKHM
         3E2H  IYSDDSVTRY SPSFQGQVTI SADKSISTAY LQWSSLKASD TAMYYCTRDG
         3E3H  IYSDDSVTRY SPSFQGQVTI SADKSISTAY LQWSSLKASD TAMYYCTRDG
         3E4H  ISYDGSNKYY ADSVKGRFTL SRDNSKNTLY LQMNSLRAED TAVYYCARED
         3E8H  ISYDGSNKYY ADSVKGRFTL PRDNSKNTLY LQMNSLRAED TAVYYCARED
         3E9H  IYSDDSVTRY SPSFQGQVTI SADKSISTAY LQWSSLKASD TAMYYCTRDG

                101                                                 150
        1CB1H  VRGVIFDYWG QGTLVTVSSA STKGPSVFPL APSSKSTSGG TAALGCLVKD
        1CC2H  VRGVIFDYWG QGTLVTVSSA STKGPSVFPL APSSKSTSGG TAALGCLVKD
         3E1H  YYG..MDVWG QGTTVTVSSA STKGPSVFPL APSSKSTSGG TAALGCLVKD
        1CC8H  VRGVIFDYWG QGTLVTVSSA STKGPSVFPL APSSKSTSGG IAALGCLVKD
        1CD7H  VRGVIFDYWG QGALVTVSSA STKGPSVFPL APSSKSTSGG TAALGCLVKD
        1CE8H  VRGVIFDYWG QGTLVTVSSA STKGPSVFPL APSSKSTSGG TAALGCLVKD
        1CC6H  VRGVLFDYWG QGTLVTVSSA STKGPSVFPL APSSKSTSGG TAALGCLVKD
         3E2H  PEA..FDIWG QGTMVTVSSA STKGPSVFPL APSSKSTSGG TAALGCLVKD
         3E3H  PEA..FDIWG QGTMVTVSSA STKGPSVFPL APSSKSTSGG TAALGCLVKD
         3E4H  YYG..MDVWG QGTTVTVSSA STKGPSVFPL APSSKSTSGG TAALGCLVKD
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          3E8H  YYG..MDVWG QGTTVTVSSA STKGPSVFPL APSSKSTSGG TAALGCLVKD
          3E9H  PEA..FDIWG QGTMVTVSSA STKGPSVFPL APSSKSTSGG TAALGCLVKD

                151                                                 200
          1CB1H  YFPEPVTVSW NSGALTSGVH TFPAVLQSSG LYSLSSVVTV PSSSLGTQTY
          1CC2H  YFPEPVTVSW NSGALTSGVH TFPAVLQSSG LYSLSSVVTV PSSSLGTQTY
          3E1H  YFPEPVTVSW NSGALTSGVH TFPAVLQSSG LYSLSSVVTV PSSSLGTQTY
          1CC8H  YFPEPVTVSW NSGALTSGVH TFPAVLQSSG LYSLSSVVTV PSSSLGTQTY
          1CD7H  YFPEPVTVSW NSGALTSGVH TFPAVLQSSG LYSLSSVVTV PSSSLGTQTY
          1CE8H  YFPEPVTVSW NSGALTSGVH TFPAVLQSSG LYSLSSVVTV PSSSLGTQTY
          1CC6H  YFPEPVTVSW NSGALTSGVH TFPAVLQSSG LYSLSSVVTV PSSSLGTQTY
          3E2H  YFPEPVTVSW NSGALTSGVH TFPAVLQSSG LYSLSSVVTV PSSSLGTQTY
          3E3H  YFPEPVTVSW NSGALTSGVH TFPAVLQSSG LYSLSSVVTV PSSSLGTQTY
          3E4H  YFPEPVTVSW NSGALTSGVH TFPAVLQSSG LYSLSSVVTV PSSSLGTQTY
          3E8H  YFPEPVTVSW NSGALTSGVH TFPAVLQSSG LYSLSSVVTV PSSSLGTQTY
          3E9H  YFPEPVTVSW NSGALTSGVH TFPAVLQSSG LYSLSSVVTV PSSSLGTQTY

                201                   227
          1CB1H  ICNVNHKPSN TKVDKKAEPK SHHHHHH
          1CC2H  ICNVNHKPSN TKVDKKAEPK SHHHHHH
          3E1H  ICNVNHKPSN TKVDKKAEPK CHHHHHH
          1CC8H  ICNVNHKPSN TKVDKKAEPK SHHHHHH
          1CD7H  ICNVNHKPSN TKVDKKAEPK SHHHHHH
          1CE8H  ICNVNHKPSN TKVDKKAEPK SHHHHHH
          1CC6H  ICNVNHKPSN TKVDKKAEPK SHHHHHH
          3E2H  ICNVNHKPSN TKVDKKAEPK CHHHHHH
          3E3H  ICNVNHKPSN TKVDKKAEPK CHHHHHH
          3E4H  ICNVNHKPSN TKVDKKAEPK CHHHHHH
          3E8H  ICNVNHKPSN TKVDKKAEPK CHHHHHH
          3E9H  ICNVNHKPSN TKVDKKAEPK CHHHHHH
```

Example 38 Growth of *E. coli* cultures and purification of recombinant antibodies and antigens

A shake flask inoculum is generated overnight from a -70 °C cell bank in an Innova 4330 incubator shaker (New Brunswick Scientific, Edison, NJ) set at 37 °C, 300 rpm. The inoculum is used to seed a 20 L fermenter (Applikon, Foster City, CA) containing defined culture medium (Pack, *et al., Bio/Technology* 11:1271 -1277 (1993)) supplemented with 3 g/L L-leucine, 3 g/L L-isoleucine, 12 g/L casein digest (Difco, Detroit, MI), 12.5 g/L glycerol and 10 mg/ml tetracycline. The temperature, pH and dissolved oxygen in the fermenter are controlled at 26 °C, 6.0-6.8 and 25 % saturation, respectively. Foam is controlled by addition of polypropylene glycol (Dow, Midland, MI). Glycerol is added to the fermenter in a fed-batch mode. Fab expression is induced by addition of L(+)-arabinose (Sigma, St. Louis, MO) to 2 g/L during the late logarithmic growth phase. Cell density is measured by optical density at 600 nm in an UV-1201 spectrophotometer (Shimadzu, Columbia, MD). Final Fab concentrations are typically 100-500 mg/L. Following run termination and adjustment of pH to 6.0, the culture is passed twice through an M-210B-EH

Microfluidizer (Microfluidics, Newton, MA) at 17000 psi. The high pressure homogenization of the cells releases the Fab into the culture supernatant.

The first step in purification is expanded bed immobilized metal affinity chromatography (EB-IMAC). Streamline Chelating resin (Pharmacia, Piscataway, NJ) is charged with 0.1 M $NiCl_2$. It is then expanded and equilibrated in 50 mM acetate, 200 mM NaCl, 10mM imidazole, 0.01% NaN_3, pH 6.0 buffer flowing in the upward direction. A stock solution is used to bring the culture homogenate to 10 mM imidazole, following which, it is diluted two-fold or higher in equilibration buffer to reduce the wet solids content to less than 5% by weight. It is then loaded onto the Streamline column flowing in the upward direction at a superficial velocity of 300 cm/hr. The cell debris passes through unhindered, but the Fab is captured by means of the high affinity interaction between nickel and the hexahistidine tag on the Fab heavy chain. After washing, the expanded bed is converted to a packed bed and the Fab is eluted with 20 mM borate, 150 mM NaCl, 200 mM imidazole, 0.01 % NaN_3, pH 8.0 buffer flowing in the downward direction. The second step in purification uses ion-exchange chromatography (IEC). Q Sepharose FastFlow resin (Pharmacia, Piscataway, NJ) is equilibrated in 20 mM borate, 37.5 mM NaCl, 0.01 % NaN_3, pH 8.0. The Fab elution pool from the EB-IMAC step is diluted four-fold in 20 mM borate, 0.01 % NaN_3, pH 8.0 and loaded onto the IEC column. After washing, the Fab is eluted with a 37.5 - 200 mM NaCl salt gradient. The elution fractions are evaluated for purity using an Xcell II SDS-PAGE system (Novex, San Diego, CA) prior to pooling. Finally, the Fab pool is concentrated and diafiltered into 20 mM borate, 150 mM NaCl, 0.01 % NaN_3, pH 8.0 buffer for storage. This is achieved in a Sartocon Slice system fitted with a 10,000 MWCO cassette (Sartorius, Bohemia, NY). The final purification yields are typically 50 %. The concentration of the purified Fab is measured by UV absorbance at 280 nm, assuming an absorbance of 1.6 for a 1 mg/mL solution.

Example 39 Generation of Cmu targeted mice

The following example describes the making of mice with disrupted, and thus non-functional, immunoglobulin genes.

Construction of a CMD targeting vector

To disrupt the mouse immunoglobulin gene, a vector containing a fragment of a murine Ig heavy chain locus is transfected into a mouse embryonic cell.

The mouse Ig heavy chain sequence "targets" the vector to the mouse immunoglobulin gene locus. The following describes construction of this immunoglobulin gene "targeting" vector.

The plasmid pICEmu contains an EcoRI/XhoI fragment of the murine Ig heavy chain locus, spanning the mu gene, that was obtained from a Balb/C genomic lambda phage library (Marcu *et al. Cell* 22: 187, 1980). This genomic fragment was subcloned into the XhoI/EcoRI sites of the plasmid pICEMI9H (Marsh et al; Gene 32, 481-485, 1984). The heavy chain sequences included in pICEmu extend downstream of the EcoRI site located just 3' of the mu intronic enhancer, to the XhoI site located approximately 1 kb downstream of the last transmembrane exon of the mu gene; however, much of the mu switch repeat region has been deleted by passage in *E. coli*.

The targeting vector was constructed as follows (*See* fig.6). A 1.3 kb HindIII/SmaI fragment was excised from pICEmu and subcloned into HindIII/SmaI digested pBluescript (Stratagene, La Jolla, CA). This pICEmu fragment extends from the HindIII site located approximately 1 kb 5' of Cmu1 to the SmaI site located within Cmu1. The resulting plasmid was digested with SmaI/SpeI and the approximately 4 kb SmaI/XbaI fragment from pICEmu, extending from the Sma I site in Cmu1 3' to the XbaI site located just downstream of the last Cmu exon, was inserted.

The resulting plasmid, pTAR1, was linearized at the SmaI site, and a neo expression cassette inserted. This cassette consists of the neo gene under the transcriptional control of the mouse phosphoglycerate kinase (pgk) promoter (XbaI/TaqI fragment; Adra *et al.* (1987) Gene 60: 65-74) and containing the pgk polyadenylation site (PvuII/HindIII fragment; Boer *et al.* (1990) Biochemical Genetics 28: 299-308). This cassette was obtained from the plasmid pKJ1 (described by Tybulewicz *et al.* (1991) *Cell* 65: 1153-1163) from which the neo cassette was excised as an EcoRI/HindIII fragment and subcloned into EcoRI/HindIII digested pGEM-7Zf (+) to generate pGEM-7 (KJ1). The neo cassette was excised from pGEM-7 (KJ1) by EcoRI/SalI digestion, blunt ended and subcloned into the SmaI site of the plasmid pTAR1, in the opposite orientation of the genomic Cmu sequences.

The resulting plasmid was linearized with Not I, and a herpes simplex virus thymidine kinase (tk) cassette was inserted to allow for enrichment of ES clones (mouse embryo-derived stem cells) bearing homologous recombinants, as described by Mansour *et al.* (1988) *Nature* 336: 348-352. This cassette consists of the coding

sequences of the tk gene bracketed by the mouse pgk promoter and polyadenylation site, as described by Tybulewicz *et al.* (1991) Cell 65: 1153-1163. The resulting CMD targeting vector contains a total of approximately 5.3 kb of homology to the heavy chain locus and is designed to generate a mutant mu gene into which has been inserted a neo expression cassette in the unique SmaI site of the first Cmu exon. The targeting vector was linearized with PvuI, which cuts within plasmid sequences, prior to electroporation into ES cells.

Generation and analysis of targeted ES cells.

The vector containing the murine Ig heavy chain gene fragment is then inserted into a mouse embryonic stem cell (an ES cell). The following describes the construction of this immunoglobulin gene-containing vector "targeted" ES cell and analysis of the ES cells' DNA after the vector has been inserted (*i.e.*, transfected) into the cell.

AB-1 ES cells (McMahon, A. P. and Bradley, A., (1990) Cell 62: 1073-1085) were grown on mitotically inactive SNL76/7 cell feeder layers (ibid.) essentially as described (Robertson, E. J. (1987) in Teratocarcinomas and Embryonic Stem Cells: a Practical Approach (E. J. Robertson, ed.) Oxford: IRL Press, p. 71-112). The linearized CMD targeting vector was electroporated into AB-1 cells by the methods described Hasty *et al.* (Hasty, P. R. *et al.* (1991) *Nature* 350: 243-246). Electroporated cells were plated into 100 mm dishes at a density of 1-2 x 106 cells/dish. After 24 hours, G418 (200 micrograms/ml of active component) and FIAU (5 x 10-7 M) were added to the medium, and drug-resistant clones were allowed to develop over 8-9 days. Clones were picked, trypsinized, divided into two portions, and further expanded. Half of the cells derived from each clone were then frozen and the other half analyzed for homologous recombination between vector and target sequences.

DNA analysis was carried out by Southern blot hybridization. DNA was isolated from the clones as described Laird *et al.* (Laird, P. W. *et al.*, (1991) *Nucleic Acids Res.* 19 : 4293). Isolated genomic DNA was digested with SpeI and probed with a 915 bp SacI fragment, probe A (figure 6), which hybridizes to a sequence between the mu intronic enhancer and the mu switch region. Probe A detects a 9.9 kb SpeI fragment from the wild type locus, and a diagnostic 7.6 kb band

from a mu locus which has homologously recombined with the CMD targeting vector (the neo expression cassette contains a SpeI site). Of 1132 G418 and FIAU resistant clones screened by Southern blot analysis, 3 displayed the 7.6 kb Spe I band indicative of homologous recombination at the mu locus. These 3 clones were further digested with the enzymes BglI, BstXI, and EcoRI to verify that the vector integrated homologously into the mu gene. When hybridized with probe A, Southern blots of wild type DNA digested with BglI, BstXI, or EcoRI produce fragments of 15.7, 7.3, and 12.5 kb, respectively, whereas the presence of a targeted mu allele is indicated by fragments of 7.7, 6.6, and 14.3 kb, respectively. All 3 positive clones detected by the SpeI digest showed the expected BglI, BstXI, and EcoRI restriction fragments diagnostic of insertion of the neo cassette into the Cmu1 exon.

Generation of mice bearing the mutated mu gene.

The three targeted ES clones, designated number 264, 272, and 408, were thawed and injected into C57BL/6J blastocysts as described by Bradley (Bradley, A. (1987) in Teratocarcinomas and Embryonic Stem Cells: a Practical Approach. (E. J. Robertson, ed.) Oxford: IRL Press, p. 113-151). Injected blastocysts were transferred into the uteri of pseudopregnant females to generate chimeric mice representing a mixture of cells derived from the input ES cells and the host blastocyst. The extent of ES cell contribution to the chimera can be visually estimated by the amount of agouti coat coloration, derived from the ES cell line, on the black C57BL/6J background. Clones 272 and 408 produced only low percentage chimeras (*i.e.* low percentage of agouti pigmentation) but clone 264 produced high percentage male chimeras. These chimeras were bred with C57BL/6J females and agouti offspring were generated, indicative of germline transmission of the ES cell genome. Screening for the targeted mu gene was carried out by Southern blot analysis of BglI digested DNA from tail biopsies (as described above for analysis of ES cell DNA). Approximately 50% of the agouti offspring showed a hybridizing BglI band of 7.7 kb in addition to the wild type band of 15.7 kb, demonstrating a germline transmission of the targeted mu gene.

Analysis of transgenic mice for functional inactivation of mu gene.

To determine whether the insertion of the neo cassette (including the Ig heavy chain sequence) into Cmu1 has inactivated the Ig heavy chain gene, a clone 264 chimera was bred with a mouse homozygous for the JHD mutation, which inactivates heavy chain expression as a result of deletion of the JH gene segments (Chen et al, (1993) Immunol. 5: 647-656). Four agouti offspring were generated. Serum was obtained from these animals at the age of 1 month and assayed by ELISA for the presence of murine IgM. Two of the four offspring were completely lacking IgM (Table 9). Genotyping of the four animals by Southern blot analysis of DNA from tail biopsies by BglI digestion and hybridization with probe A (figure 6), and by StuI digestion and hybridization with a 475 bp EcoRI/StuI fragment (ibid.) demonstrated that the animals which fail to express serum IgM are those in which one allele of the heavy chain locus carries the JHD mutation, the other allele the Cmu1 mutation. Mice heterozygous for the JHD mutation display wild type levels of serum Ig. These data demonstrate that the Cmu1 mutation inactivates expression of the mu gene.

TABLE 9

Mouse	Serum IgM (micrograms/ml)	Ig H chain genotype
42	<0.002	CMD/JHD
43	196	+/JHD
44	<0.002	CMD/JHD
45	174	+/JHD
129 x BL6 F1	153	+/+
JHD	<0.002	JHD/JHD

Table 2. Level of serum IgM, detected by ELISA, for mice carrying both the CMD and JHD mutations (CMD/JHD), for mice heterozygous for the JHD mutation (+/JHD), for wild type (129Sv x C57BL/6J)F1 mice (+/+), and for B cell deficient mice homozygous for the JHD mutation (JHD/JHD).

Example 40 Generation of HCo12 transgenic mice

The following describes the generation of transgenic mice containing human immunoglobulin heavy chain gene sequence that can generate human immunoglobulins. Because these mice cannot make endogenous (i.e., mouse) immunoglobulins, upon challenge with antigen, *e.g.*, a human polypeptide, only human sequence immunoglobulins are made by the transgenic mouse.

The HCo12 human heavy chain transgene.

The HCo12 transgene was generated by coinjection of the 80 kb insert of pHC2 (Taylor *et al.*, 1994, Int. Immunol., 6: 579-591) and the 25 kb insert of pVx6. The plasmid pVx6 was constructed as described below. An 8.5 kb HindIII/SalI DNA fragment, comprising the germline human VH1-18 (DP-14) gene together with approximately 2.5 kb of 5' flanking, and 5 kb of 3' flanking genomic sequence was subcloned into the plasmid vector pSP72 (Promega, Madison, WI) to generate the plasmid p343.7.16. A 7 kb BamHI/HindIII DNA fragment, comprising the germline human VH5-51 (DP-73) gene together with approximately 5 kb of 5' flanking and 1 kb of 3' flanking genomic sequence, was cloned into the pBR322 based plasmid cloning vector pGP1f (Taylor *et al.* 1992, Nucleic Acids Res. 20: 6287-6295), to generate the plasmid p251f.

A new cloning vector derived from pGP1f, pGP1k (Seq. ID #1), was digested with EcoRV/BamHI, and ligated to a 10 kb EcoRV/BamHI DNA fragment, comprising the germline human VH3-23 (DP47) gene together with approximately 4 kb of 5' flanking and 5 kb of 3' flanking genomic sequence. The resulting plasmid, p112.2RR.7, was digested with BamHI/SalI and ligated with the 7 kb purified BamHI/SalI insert of p251f. The resulting plasmid, pVx4, was digested with XhoI and ligated with the 8.5 kb XhoI/SalI insert of p343.7.16.

A plasmid clone was obtained with the V_H1-18 gene in the same orientation as the other two V genes. This clone, designated pVx6, was then digested with NotI and the purified 26 kb insert coinjected--together with the purified 80 kb NotI insert of pHC2 at a 1:1 molar ratio--into the pronuclei of one-half day (C57BL/6J x DBA/2J)F2 embryos as described by Hogan *et al.* (B. Hogan *et al.*, Manipulating the Mouse Embryo, A Laboratory Manual, 2nd edition, 1994, Cold Spring Harbor Laboratory Press, Plainview NY).

Three independent lines of transgenic mice comprising sequences from both Vx6 and HC2 were established from mice that developed from the injected embryos. These lines of transgenic mice are designated (HCo12)14881, (HCo12)15083, and (HCo12)15087. Each of the three lines were then bred with mice comprising the CMD mutation described in Example 23, the JKD mutation (Chen *et al.* 1993, EMBO J. 12: 811-820), and the (KCo5)9272 transgene (Fishwild *et al.* 1996, *Nature* Biotechnology 14: 845-851). The resulting mice express human heavy

and kappa light chain transgenes (and produce human sequence heavy and kappa light chain antibodies) in a background homoygous for disruption of the endogenous mouse heavy and kappa light chain loci.

Two different strains of mice were used to generate hybridomas and monoclonal antibodies reactive to human IL-8. Strain ((CMD)++; (JKD)++; (HCo7)11952+/++; (KCo5)9272+/++), and strain ((CMD)++; (JKD)++; (HCo12)15087+/++; (KCo5)9272+/++). Each of these strains are homozygous for disruptions of the endogenous heavy chain (CMD) and kappa light chain (JKD) loci. Both strains also comprise a human kappa light chain transgene (HCo7), with individual animals either hemizygous or homozygous for insertion #11952. The two strains differ in the human heavy chain transgene used. Mice were hemizygous or homozygous for either the HCo7 or the HCo12 transgene. The CMD mutation is described above in Example 23, above. The generation of (HCo12)15087 mice is described above. The JKD mutation (Chen *et al.* 1993, EMBO J. 12: 811-820) and the (KCo5)9272 (Fishwild *et al.* 1996, *Nature* Biotechnology 14: 845-851) and (HCo7)11952 mice, are described in US patent 5,770,429 (Lonberg & Kay, 6/23/98).

WHAT IS CLAIMED IS:

1. A method of producing a human antibody display library, comprising:

providing a nonhuman transgenic animal whose genome comprises a plurality of human immunoglobulin genes that can be expressed to produce a plurality of human antibodies;

isolating a population of nucleic acids encoding human antibody chains from lymphatic cells of the nonhuman transgenic animal;

forming a library of display packages displaying the antibody chains, wherein a library member comprises a nucleic acid encoding an antibody chain, and the antibody chain is displayed from the package.

2. The method of claim 1, further comprising:

contacting libraries members with a target, whereby library members displaying an antibody chain and binding partner (if present) with specific affinity for the target bind to the target, to produce a subpopulation of display packages;

wherein the subpopulation of display packages comprises at least ten different display packages comprising at least ten nucleic acids encoding at least ten antibody chains, and at least 50% of the nucleic acids encode human antibody chains, which with the binding partner (if present) show at least 10^8 M^{-1} affinity for the target and no library member constitutes more than 50% of the library.

3. The method of claim 1, further comprising preparing a subpopulation of the isolated lymphatic cells enriched for lymphatic cells expressing an IgG antibody before the isolating step.

4. The method of claim 1, wherein the subpopulation is prepared by contacting the isolated lymphatic cells with a reagent that binds to the Fc region of an IgG antibody.

5. The method of claim 1, wherein the isolating step comprises PCR amplification using a pair of primers one of which is specific for DNA encoding IgG heavy chains.

6. The method of claim 2, wherein the at least 50% of the nucleic acids have a median of at least 2 somatic mutations per antibody chain encoded by the nucleic acids.

7. The method of claim 2, wherein the at least 50% of the nucleic acid have a median of at least 5 somatic mutations per antibody chain encoded by the nucleic acids.

9. The method of claim 1, wherein the lymphatic cells are obtained from bone marrow.

10. The method of claim 1, wherein the lymphatic cells are from a nonhuman transgenic animal that has been immunized with an immunogen without developing a titer to the immunogen greater than ten fold of a negative control.

11. The method of claim 1, wherein the lymphatic cells are from a nonhuman transgenic animal that has been immunized with an immunogen without developing a detectable titer against the immunogen.

12. The method of claim 2, wherein the target is expressed on the surface of a cell.

13. The method of claim 2, wherein the target is a protein within a phospholipid membrane or particle.

14. The method of claim 2, wherein at least 90% of the human antibody chains have IgG isotype..

15. The method of claim 1, further comprising producing RNA transcripts of the nucleic acids, and translating the transcripts to form antibody chains under conditions in which an antibody chain remains linked to the RNA transcript from which the antibody chain was translated, the complex formed between the transcript and the antibody chain constituting a library member.

16. The method of claim 1, further comprising cloning the population of nucleic acids into multiple copies of a phage display vector and expressing the vector in host cells to form the library of display packages.

17. The method of claim 16, wherein the phage display vector is a phagemid vector.

18. The method of claim 1, wherein the nucleic acids encode variable regions of the antibody chains and the display vector comprises a segment encoding a human constant region and the cloning joins a nucleic acid encoding a variable region in-frame with the segment encoding the human constant region.

19. The method of claim 18, wherein the antibody chain is a heavy chain and the constant region comprises a C_H1 region.

20. The method of claim 18, wherein the antibody chain is a light chain and the constant region comprises a C_κ or C_λ constant region.

21. The method of claim 1, wherein the antibody chain comprises a heavy or light chain which in at least some library members is complexed to a binding partner, comprising respectively a partner light or heavy human chain to form a Fab fragment.

22. The method of claim 1, further comprising immunizing the nonhuman transgenic animal with an antigen.

23. The method of claim 22, further comprising hyperimmunizing the animal.

24. The method of claim 23, wherein the antigen is the target or an immunogenic fragment thereof.

25. The method of claim 1, wherein a library member further comprises a nucleic acid segment encoding a tag linked to the nucleic acid encoding the antibody chain, wherein the tag is the same in different library members.

26. The method of claim 25, further comprising contacting library members with a receptor having specific affinity for the tag and isolating a subpopulation of library members that bind to immobilized receptor.

27. The method of claim 26, further comprising contacting the subpopulation of library members that bound to the immobilized receptor with a target lacking specific affinity for the tag, and isolating a further subpopulation of library members that binds to the target.

28. The method of claim 27, further comprising subcloning en masses nucleic acids encoding antibody chains from the further subpopulation of library members into multiple copies of an expression vector to form modified expression vectors.

29. The method of claim 28, further comprising expressing the modified expression vectors in host cells to produce a library of human antibody chains.

30. A method of producing a human Fab phage display library, comprising:

providing a nonhuman transgenic animal whose genome comprises a plurality of human immunoglobulin genes that can be expressed to produced a plurality of human antibodies;

isolating populations of nucleic acids respectively encoding human antibody heavy chains and human antibody light chains from lymphatic cells of the nonhuman transgenic animal;

cloning the populations into multiple copies of a phage display vector to produce a display library, wherein a library member comprises a phage capable of displaying from its outersurface a fusion protein comprising a phage coat protein, a human antibody light chain or human antibody heavy chain , wherein in at

least some members, the human antibody heavy or light chain is complexed with a partner human antibody heavy or light chain, , the complex forming a Fab fragment to be screened.

31. The method of claim 30, further comprising:

contacting libraries members with a target, whereby library members displaying a complex of a human heavy and light chain with specific affinity for the target bind to the target, to produce a subpopulation of display packages;

wherein the subpopulation of display packages comprises at least ten different display packages comprising at least ten pairs of nucleic acids encoding at least ten pairs of heavy and light chains, and at least 50% of the pairs of nucleic acids encoding pairs of heavy and light chains forming complexes showing at least 10^8 M^{-1} affinity for the target and no library member constitutes more than 50% of the library.

32. The method of claim 31, further comprising preparing a subpopulation of the isolated lymphatic cells enriched for lymphatic cells expressing an IgG antibody.

33. The method of claim 31, wherein the subpopulation is prepared by contacting the isolated lymphatic cells with a reagent that binds to the Fc region of an IgG antibody.

34. The method of claim 31, wherein the isolating step comprises PCR amplification using a pair of primers one of which is specific for DNA encoding IgG heavy chains.

35. The method of claim 30, wherein the at least 50% of the pairs of nucleic acids have a median of at least 10 mutations in the nucleic acids encoding heavy chains and a median of at least two somatic mutations in the nucleic acids encoding light chains.

36. The method of claim 31, wherein the at least 50% of the pairs of nucleic acids have a median of at least 10 somatic mutations in the nucleic acids encoding the heavy chains and at least five somatic mutations in the nucleic acids encoding the light chains.

37. The method of claim 31, wherein the at least 50% of the pairs of nucleic acids having a median of at least ten somatic mutations in the nucleic acids encoding the heavy chains and a median of at least ten somatic mutations in the nucleic acids encoding the light chains.

38. The method of claim 31, wherein the lymphatic cells are obtained from bone marrow.

39. The method of claim 31, wherein the lymphatic cells are from a nonhuman transgenic mammal that has been immunized with an immunogen without developing a significant titer to the immunogen.

40. The method of claim 31, wherein the target is expressed on the surface of a cell.

41. The method of claim 31, wherein at least 90% of the human antibody chains have IgG isotype..

42. The method of claim 31, wherein the plurality of human genes is free of human lambda light chain genes.

43. The method of claim 31, wherein there are no more than 40 human VH genes included in the plurality of human genes.

44. The method of claim 31, wherein there are no more than 40 human VL genes included in the plurality of human genes.

45. The method of claim 31, wherein each copy of the phage

display vector receives a random combination of nucleic acids encoding heavy and light chains from the respective populations.

46. The method of claim 31, wherein the populations of nucleic acids respectively encode populations of human heavy and light chain variable regions, and the phage display vector encodes human heavy and light chain constant regions expressed in frame with human heavy and light chains inserted into the vector.

47. The method of claim 31, further comprising contacting libraries members from the sublibrary with a target, whereby library members displaying a Fab fragment with specific affinity for the target bind to the target, and separating phage displaying Fab fragments bound to the target to produce a further subpopulation of phage.

48. The method of claim 31, further comprising isolating a phage displaying a Fab fragment that binds to the target.

49. The method of claim 31, further comprising immunizing the nonhuman transgenic animal with an antigen.

50. The method of claim 31, further comprising expressing a Fab fragment from a phage bound to the target in soluble form.

51. The method of claim 30, wherein the fusion protein further comprises a tag that is the same in different library members; and the method further comprises contacting the library members with a receptor that specifically binds to the tag, and isolating library members that bind to the tag..

52. The method of claim 51, further comprising contacting the library members that bind to the tag with a target lacking specific affinity for the tag, and isolating a further subpopulation of library members bound to the target.

53. The method of claim 52, further comprising subcloning a mixed population of nucleic acids encoding human antibody heavy chains and human antibody light chains from the further subpopulation of library members into multiple copies of an expression vector to produce modified expression vectors.

54. The method of claim 53, further comprising expressing the modified expression vectors in host cells to produce a population of human antibodies.

55. The method of claim 54, wherein the population of human antibodies includes at least 10 different antibodies.

56. The method of claim 54, wherein the population of human antibodies includes at least 100 different antibodies.

57. The method of claim 54, wherein the population of human antibodies includes at least 1000 different antibodies.

58. A library of at least ten different nucleic segments encoding human antibody chains, wherein at least 50% of segments in the library encode human antibody chains showing at least 10^8 M^{-1} affinity for the same target and no library member constitutes more than 50% of the library.

59. The library of claim 58, wherein the library comprises at least ten pairs of different nucleic acid segments, the members of a pair respectively encoding heavy and light human antibody chains, wherein at least 50% of the pairs encode heavy and light human antibody chains that form complexes showing specific affinity for the same target, and no pair of nucleic acid segments constitutes more than 50% of the library.

60. The library of claim 59, wherein the library comprises at least 100 pairs of different nucleic acid segments.

61. The library of claim 59, wherein the library comprises at least 1000 pairs of different nucleic acid segments.

62. The library of claim 59, wherein at least at least 50% of the pairs encode heavy and light chains that form complexes having affinity of at least 10^9 M^{-1} for the target.

63. The library of claim 59, wherein at least 50% of the pairs encode heavy and light chains that form complexes having affinity of at least 10^{10} M^{-1} for the target.

64. The library of claim 59, wherein at least 90% of the pairs of different nucleic acid segments encode heavy and light chains that form complexes having at least 10^9 M^{-1} affinity of the target.

65. The library of claim 59, wherein the at least 90% of the pairs of different nucleic acid segments have a median of at least 10 somatic mutations in the nucleic acids encoding the heavy chains and a median of at least 2 somatic mutations in the nucleic acids encoding the light chains.

66. The library of claim 59, wherein the at least 90% of the pairs of different nucleic acid segments have a median of at least 10 somatic mutations in the nucleic acids encoding the heavy chains and a median of at least 10 somatic mutations in the nucleic acids encoding the light chains.

67 The library of claim 59 wherein at least 90% of pairs of different nucleic acids segments have a nucleic acid segment encoding a heavy chain of IgG isotype.

68 A library of at least ten different nucleic segments encoding human antibody chains, wherein at least 90% of segments in the library encode human antibody chains for the same target and no library member constitutes more than 50% of the library, and the library is free of segments encoding human lambda light chains.

69 A library of at least 1000 different nucleic segments encoding human antibody chains, wherein at least 90% of segments in the library encode human antibody chains for the same target and no library member constitutes more than 50% of the library, wherein each segment comprises subsequence(s) from a human VH and/or a human VL gene, and no more than 40 human VH genes and no more than 40 human VL genes are represented in the library.

70. A library of at least ten types of human antibodies, wherein at least 50% of the types of human antibodies in the library have an affinity of at least 10^{10} M^{-1} for the same target and no type of library member constitutes more than 25% of the library.

71. The library of claim 70 having at least 100 different types of human antibody.

72. The library of claim 71, wherein the at least 50% of the types of human antibodies in the library have an affinity of at least 10^{11} M^{-1}.

73. The library of claim 72, wherein the at least 50% of the types of human antibodies in the library have an affinity of at least 10^{12} M^{-1}.

74. A method of producing a human antibody display library, comprising:

introducing an immunogen into a nonhuman transgenic animal whose genome comprises a plurality of human immunoglobulin genes that can be expressed to produce a plurality of human antibodies;

isolating a population of nucleic acids encoding human antibody chains from lymphatic cells of the nonhuman transgenic animal, wherein the nonhuman transgenic animal lacks a titer to the immunogen greater than ten fold the background titer before immunization;

forming a library of display packages displaying the antibody chains, wherein a library member comprises a nucleic acid encoding an antibody chain, and the antibody chain is displayed from the package.

75. The method of claim 74, wherein the nonhuman transgenic animal lacks a detectable titer to the immunogen when the isolating step is performed.

76. The method of claim 74, wherein the immunogen is a nucleic acid.

77. The method of claim 76, wherein the nucleic acid encodes a membrane bound receptor.

78. The method of claim 76, further comprising

contacting libraries members with a target, whereby library members displaying an antibody chain and binding partner (if present) with specific affinity for the target bind to the target, to produce a subpopulation of display packages;

wherein the subpopulation of display packages comprises at least ten different display packages comprising at least ten nucleic acids encoding at least ten antibody chains, and at least 50% of the nucleic acids encode human antibody chains, which in combination with the binding partner (if present) show at least 10^{10} M^{-1} affinity for the target and no library member constitutes more than 50% of the library

79. The method of claim 78, wherein the at least 50% of the nucleic acid encoding human antibody chains, in combination with a binding partner (if present) show at least $10^{12} M^{-1}$ affinity for the target.

80. A method of producing a human antibody display library, comprising:

providing a nonhuman transgenic animal whose genome comprises a plurality of human immunoglobulin genes that can be expressed to produce a plurality of human antibodies;

obtaining lymphatic cells from the nonhuman mammal and enriching the cells to produce a subpopulation enriched for cells expressing antibodies of IgG isotype

isolating populations of nucleic acids encoding human heavy and light antibody chains from the subpopulation;

forming a library of display packages displaying the human heavy and light antibody chains, wherein a library member comprises nucleic acids encoding human antibody heavy and light chains, and a complex of the heavy and light chains is displayed from the library member

81. The method of claim 80, wherein at least 90% of library members comprises a nucleic acid encoding an antibody chain with IgG isotype.

82. The method of claim 80, wherein the nucleic acids encoding the human antibody heavy chains and the nucleic acids encoding the human antibody light chains both have a median of at least 5 somatic mutations per nucleic acid.

83. A method of producing a human antibody display library, comprising:

introducing a nucleic acid encoding a protein immunogen into a nonhuman transgenic animal whose genome comprises a plurality of human immunoglobulin genes that can be expressed to produce a plurality of human antibodies,

isolating a population of nucleic acids encoding human antibody chains from lymphatic cells of the nonhuman transgenic animal;

forming a library of display packages displaying the antibody chains, wherein a library member comprises a nucleic acid encoding an antibody chain, and the antibody chain is displayed from the package.

84. the method of claim 83, wherein the protein immunogen is a natural protein.

85. The method of claim 83, wherein the natural protein is a natural human protein.

86. The method of claim 83, wherein the nucleic acid encodes a membrane bound protein.

87. The method of claim 83, wherein the nucleic acid encodes an EST.

88. The method of claim 83, further comprising

contacting libraries members with a target, whereby library members displaying an antibody chain and binding partner (if present) with specific affinity for the target bind to the target, to produce a subpopulation of display packages;

wherein the subpopulation of display packages comprises at least ten different display packages comprising at least ten nucleic acids encoding at least ten antibody chains, and at least 50% of the nucleic acids encode human antibody chains, which in combination with a binding partner (if present) show at least 10^{10} M^{-1} affinity for the target and no library member constitutes more than 50% of the library

89. The method of claim 88, wherein the at least 50% of the nucleic acid encoding human antibody chains, in combination with a binding partner (if present) show at least 10^{12} M^{-1} affinity for the target.

90. A method of preparing a population of antibodies comprising:

screening a first library of display packages displaying antibody chains, wherein a library member comprises a nucleic acid encoding an antibody chain, and the antibody chain is displayed from the package for binding to a target to

isolate a first population of display packages displaying antibody chains that specifically bind to the target;

screening a second library of display packages displaying antibody chains, wherein a library member comprises a nucleic acid encoding an antibody chain, and the antibody chain is displayed from the package for binding to the target, wherein the screening is conducted in the presence of antibodies displayed from the first population of display packages to generate a second population of display packages displaying antibody chains that specifically bind to the target,

whereby the antibody chains in the second population of chains and the antibody chains in the first population of chain have different epitope binding profiles in the target.

91. The method of claim 90, further comprising subcloning the nucleic acids encoding antibody chains into the first and/or second library of display members from lymphatic cells of a nonhuman transgenic animal whose genome comprises a plurality of human immunoglobulin genes that can be expressed to produce a human antibody to the protein encoded by the nucleic acid.

92. The use of a nucleic acid to immunize a nonhuman transgenic animal whose genome comprises a plurality of human immunoglobulin genes that can be expressed to produce a human antibody to the protein encoded by the nucleic acid.

93. The use of claim 92, wherein the nucleic acid encodes a natural human protein.

94. The use of an immunized animal that lacks a detectable titer to the immunogen for the production of antibodies to the immunogen.

95. The use of claim 94, wherein the animal is a nonhuman transgenic animal whose genome comprises a plurality of human immunoglobulin genes for the production of human antibodies to the immunogen.

96. The use of a display method to screen nucleic acids encoding antibody chains obtained from an immunized nonhuman transgenic animal whose

genome comprises a plurality of human immunoglobulin genes to produce a highly enriched polyclonal population of human antibodies with high affinity for the immunogen.

97. The use of enrichment of a population of B cells for a subpopulation expressing antibodies of IgG isotype for the production of a display library containing random combinations of heavy and light chains.

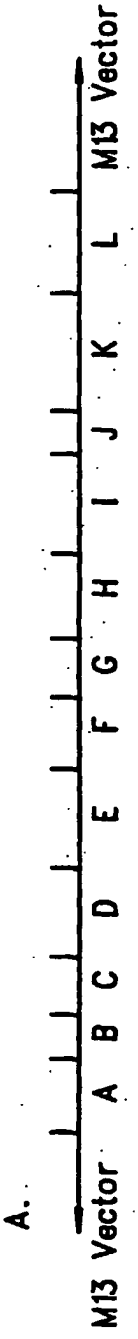


FIG. 1

DNA Sequences of Oligos used to delete CDRI-CDR3 regions of 668-4

Kappa Chain

Framework 4 Stop Stop Stop Framework 1

TAT TTC CAG CTT GGT CCC TCT AGA GTT AAC GAT ATC AA CGT TTA T CTA A TCA GCA AGA GAT GGA GGC TTG

Heavy Chain

Framework 4 Stop Stop Stop Framework 4

TGA GGT TCC TTG ACC CCA CTG CAG AGT ACT AGG CCT CT GAG CTA C TCA G TTA GGT GAT TGA GTA GCC AGT

FIG. 2

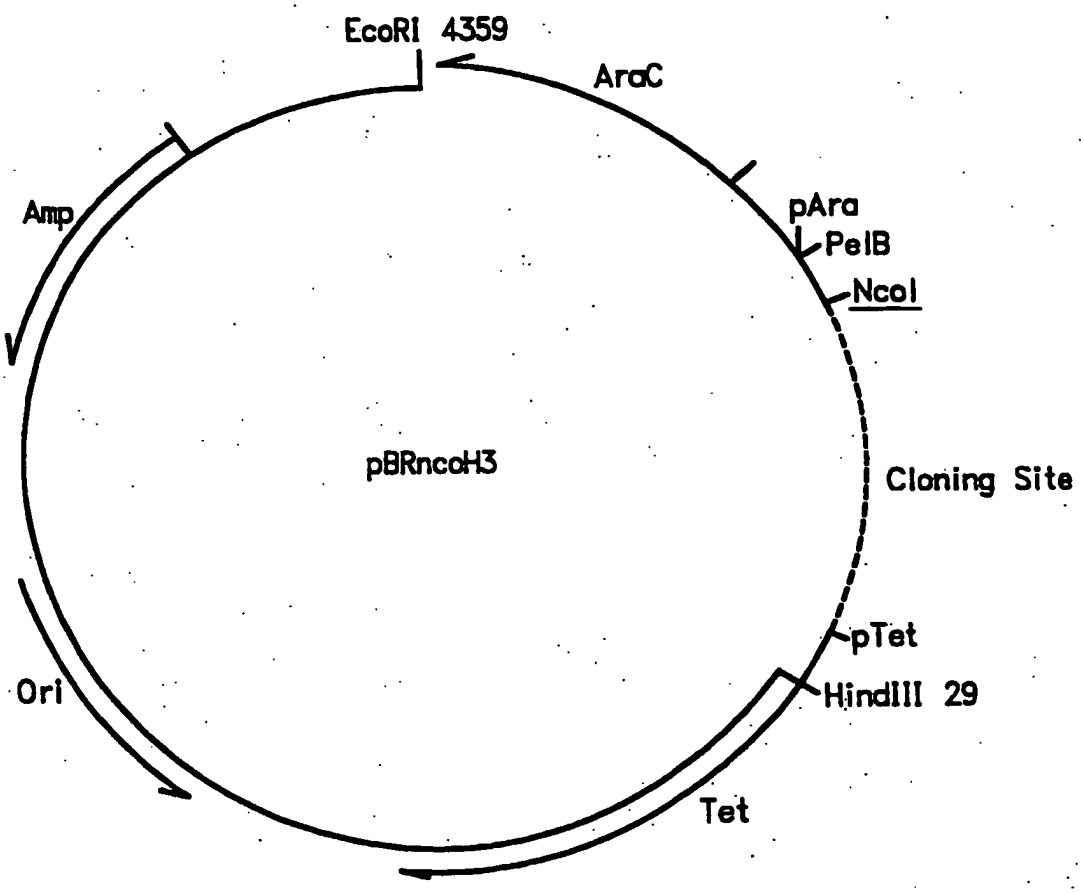

EcoRI 4359
AraC
pAra
PelB
NcoI
Cloning Site
pTet
HindIII 29
Tet
Ori
Amp
pBRncoH3

FIG. 3

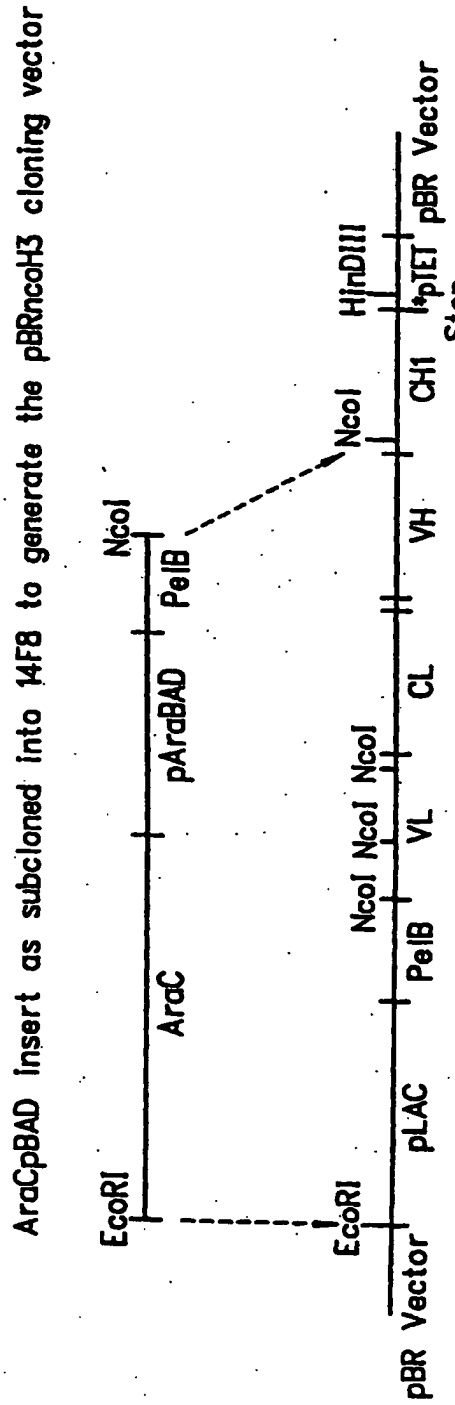
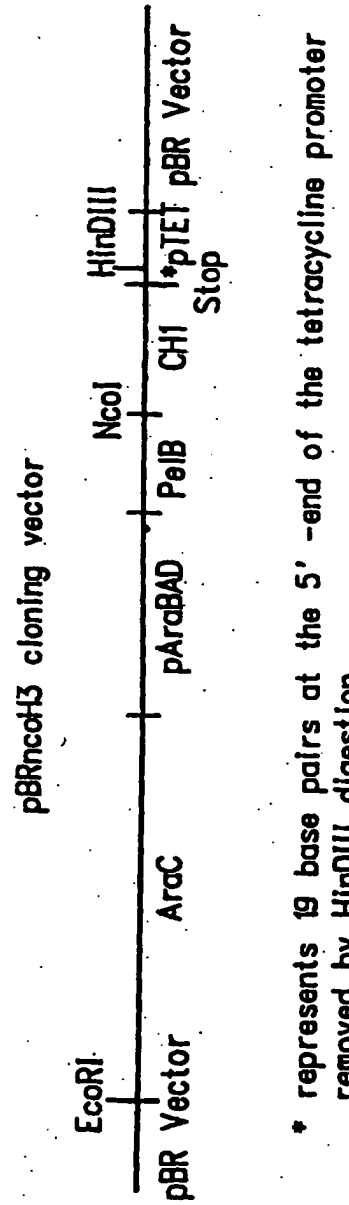


FIG. 4A



* represents 19 base pairs at the 5' -end of the tetracycline promoter removed by HinDIII digestion

FIG. 4B

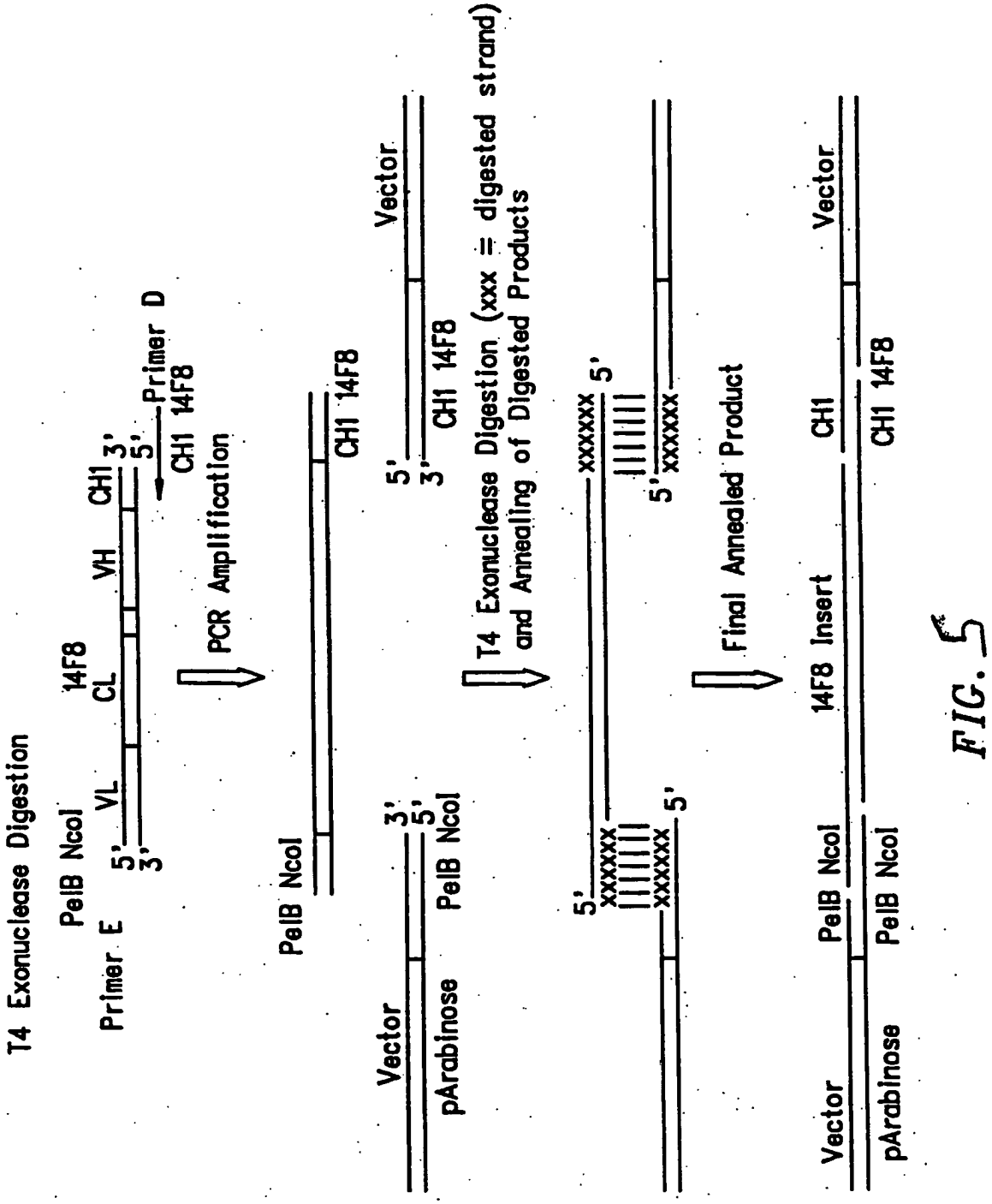
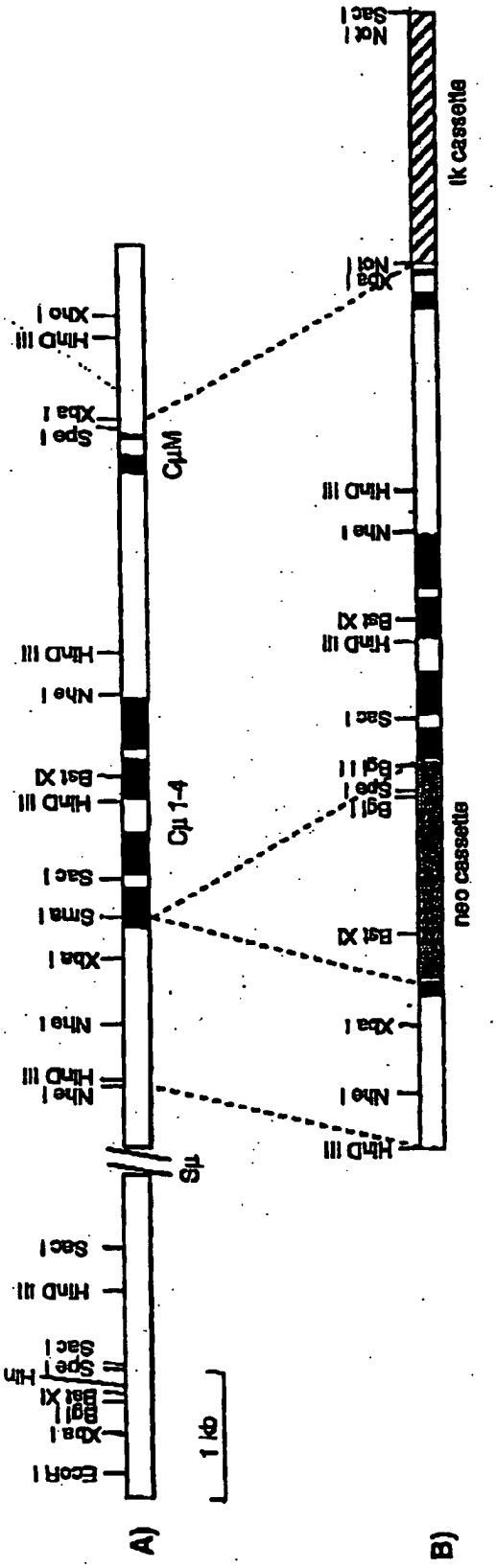

T4 Exonuclease Digestion
PelB NcoI
14F8
Primer E
CL
VL
VH
CH1
3'
5'
Primer D
CH1 14F8
PCR Amplification
PelB NcoI
CH1 14F8
Vector
pArabinose
T4 Exonuclease Digestion (xxx = digested strand)
and Annealing of Digested Products
xxxxxx
Final Annealed Product
14F8 Insert

FIG. 5



Expected Fragment Sizes (kb) using Probe A		
Restriction Digest	Fragment Length wild type	mutant
Bgl I	15.7	7.7
Bst XI	7.3	6.6
Spe I	9.9	7.6
Eco RI	12.5	14.3

Fig. 6 Targeted insertion of a neo cassette into the Sma I site of the μ1 exon. A) Schematic diagram of the genomic structure of the μ locus. The filled boxes represent the μ exons. B) Schematic diagram of the CmD targeting vector. The dotted lines denote those genomic μ sequences included in the construct. Plasmid sequences are not shown. C) Schematic diagram of the targeted μ locus in which the neo cassette has been inserted into μ1. The box at the right shows those RFLP's diagnostic of homologous recombination between the targeting construct and the μ locus. The RFLP's were detected by Southern blot hybridization using probe A, the 915 bp Sac I fragment shown in diagram C.

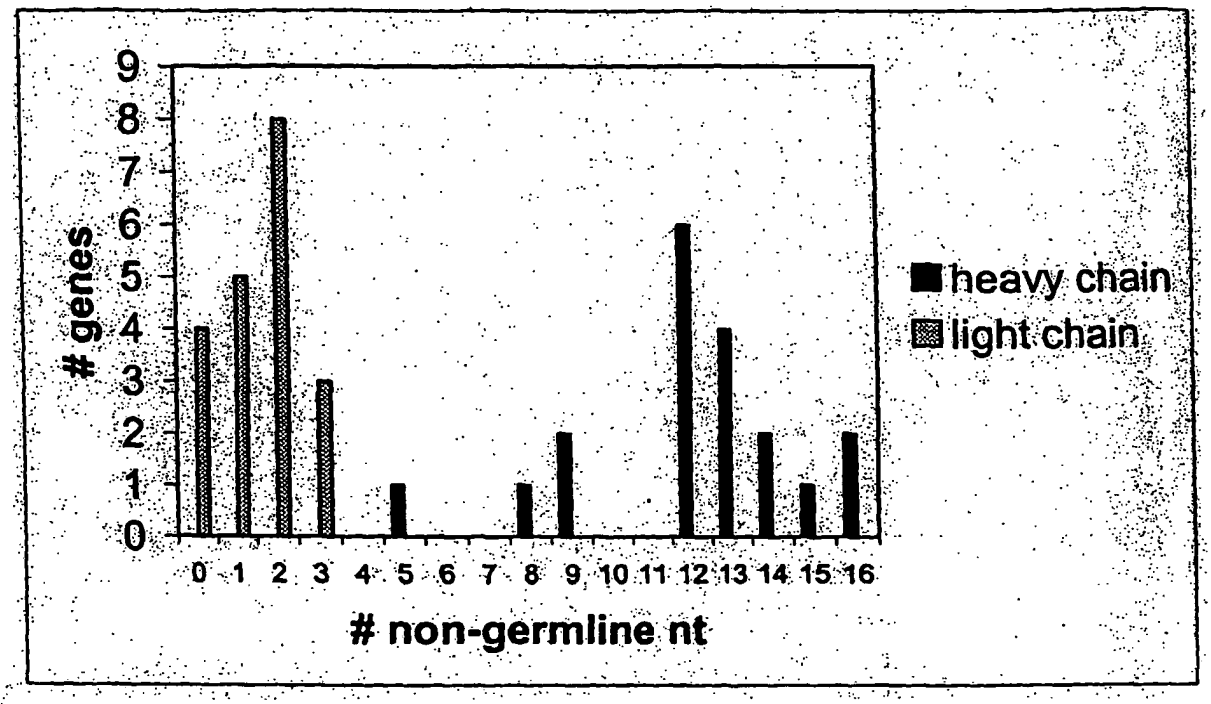

9
8
7
6
5
4
3
2
1
0
genes
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
non-germline nt
heavy chain
light chain

Figure 7.

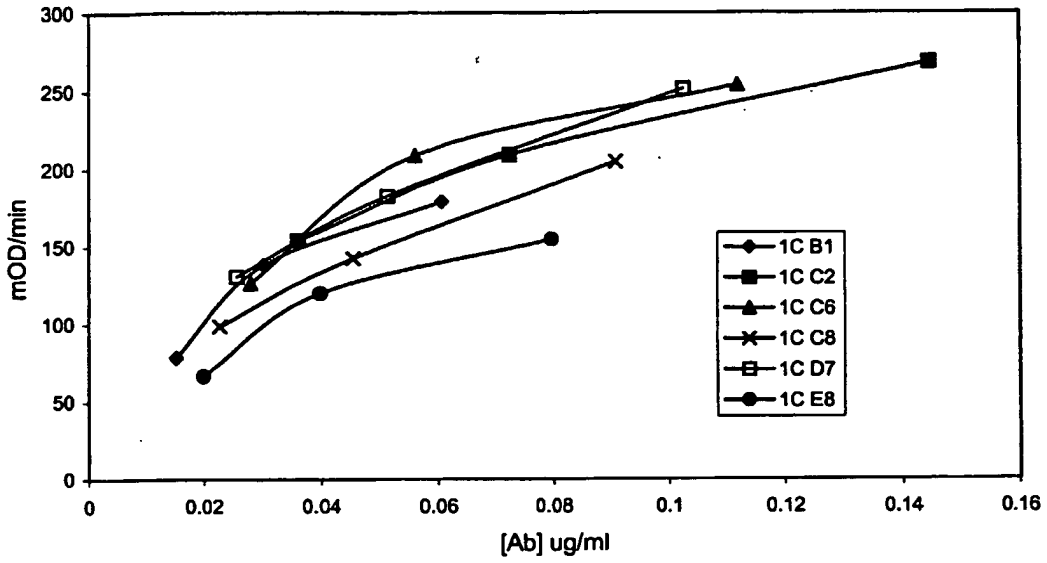

300
250
200
150
100
50
0
mOD/min
0
0.02
0.04
0.06
0.08
0.1
0.12
0.14
0.16
[Ab] ug/ml
1C B1
1C C2
1C C6
1C C8
1C D7
1C E8

Fig. 8

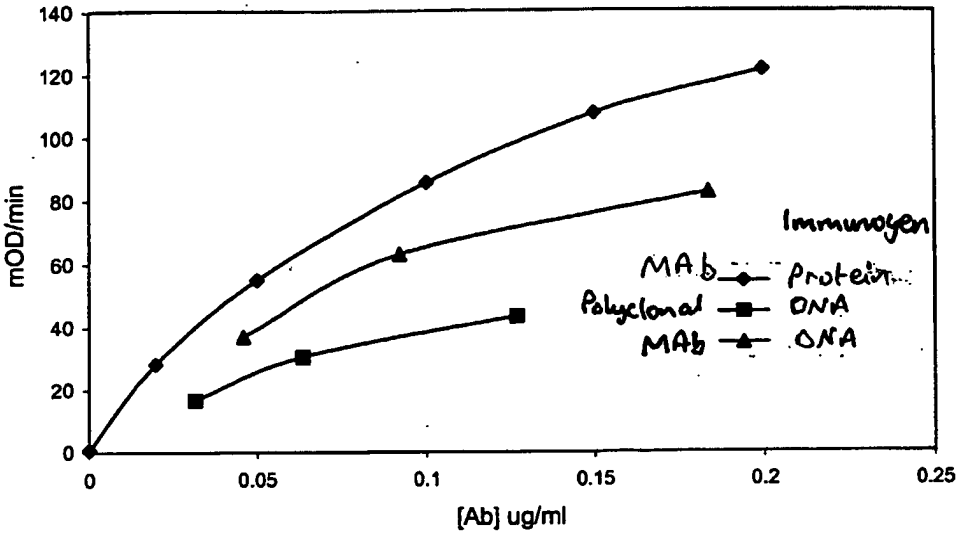

140
120
100
80
60
40
20
0
mOD/min
0
0.05
0.1
0.15
0.2
0.25
[Ab] ug/ml
Immunogen
MAb Protein
Polyclonal DNA
MAb DNA

Fig. 9

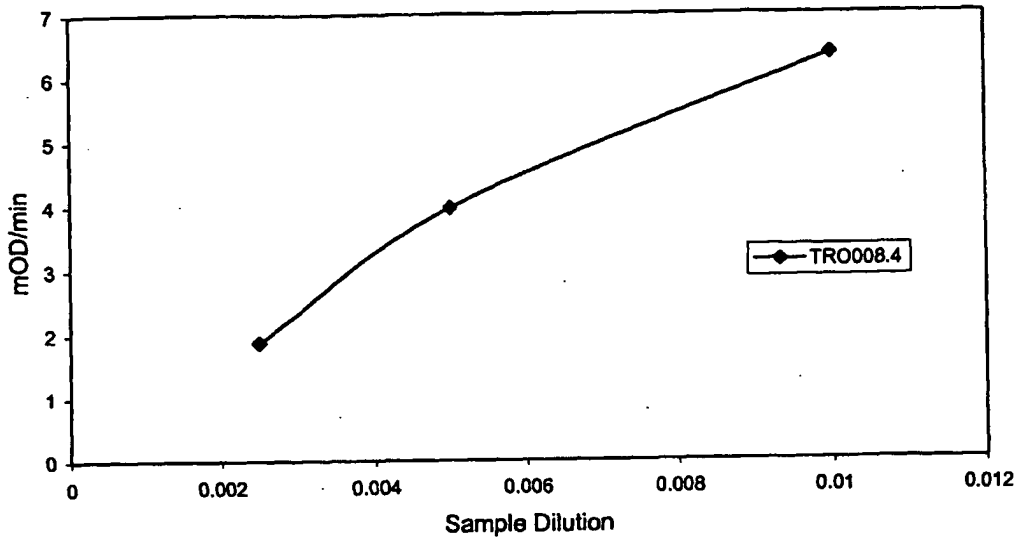

7
6
5
4
3
2
1
0
mOD/min
0
0.002
0.004
0.006
0.008
0.01
0.012
Sample Dilution
TRO008.4

Fig. 10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/27237

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/5, 6, 7.1, 91.4, 91.5, 69.1, 320.1, 326, 471, 489; 536/23.1, 23,4, 23,53, 24.3; 800/13, 14, 22

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y, P	US 6,057,098 A (BUECHLER et al) 02 May 2000, see the entire document, especially col. 2-4; col. 5, lines 1-6; col. 18, lines 39-62.	1-97
X, P --- Y, P	WO 99/53049 A1 (ABGENIX, INC.) 21 October 1999, see the entire document, especially page 28, lines 1-12.	92-93 --- 1-91, 94-97
Y	WO 98/47343 A2 (BIOSITE DIAGNOSTICS, INC.) 29 October 1998, see the entire document, especially pages 3-7.	1-97
Y	US 5,770,429 A (LONBERG et al) 23 June 1998, see the entire document.	1-57, 74-89, 92-96
Y	WO 96/33735 A1 (CELL GENESYS, INC.) 31 October 1996, see the entire document, especially page 11, lines 15-35.	1-97

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 19 JANUARY 2001	Date of mailing of the international search report 01 MAR 2001
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer QUANG NGUYEN, PH.D. Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/27237

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 94/26787 A1 (THE BOARD OF TRUSTEES OF THE LELAND STANFORD JUNIOR UNIVERSITY) 24 November 1994, page 6, lines 25-29.	90-91
A	RADER et al. Phage display of combinatorial antibody libraries. Current Opinion in Biotechnology. 01 August 1997, Vol. 8, pages 503-508.	1-97

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/27237

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/27237

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (7):

C12Q 1/70, 1/68; G01N 33/53; C12N 15/64, 15/00, 15/74, 5/06, 5/16; C12P 19/34, 21/06; C07H 21/04; A01K 67/00, 67/027

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/5, 6, 7.1, 91.4, 91.5, 69.1, 320.1, 326, 471, 489; 536/23.1, 23,4, 23,53, 24.3; 800/13, 14, 22

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, DIALOG, MEDLINE, BIOSIS, EMBASE
Search terms: human antibody, antibody display library, phage display library, transgenic animal, human immunoglobulin gene, xenomouse

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claims 1-91, drawn to a method of producing a human antibody display library and a library generated.
Group II, claims 92-93, drawn to the use of a nucleic acid to immunize a nonhuman transgenic animal.
Group III, claims 94-95, drawn to the use of an immunized animal that lacks a detectable tier to the immunogen for the production of antibodies to the immunogen.
Group IV, claim 96, drawn to the use of a display method to screen nucleic acids encoding antibody chains obtained from an immunized nonhuman transgenic animal.
Group V, claim 97, drawn to the use of enrichment of a population of B cells for a subpopulation expressing antibodies of IgG isotype for the production of a display library.

The inventions listed as Groups I-V do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: they are multiple independent methods.